REMARKS

Claims 1, 2, 10 and 11 are pending in the application. Applicants herewith amend claims 1 and 10, support for which is found at least at pages 4 to 9 of the specification. Applicants cancel claim 2. No new matter is added. Entry of the amendment is kindly requested.

Applicants thank the Examiner for withdrawal of the objections and/or rejections listed at page 4 of the Office Action dated February 12, 2007. Applicants thank the Examiner for acknowledging the Information Disclosure Statement filed on October 24, 2006. There are no outstanding Information Disclosure Statements at this time.

I. Claim 2 is Cancelled

At page three of the Office Action, the Examiner objects to Claim 2. Claim 2 is herewith cancelled therefore, the objection to claim 2 is moot.

II. Claims 1, 10 and 11 are Patentable Under 35 U.S.C. § 101 and § 112 first paragraph

At page three of the Office Action, the Office rejects claims 1, 2, 10 and 11 under 35 U.S.C. § 101 as allegedly lacking utility. At page 9 of the Office Action, the Office rejects claims 1, 2, 10 and 11 under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement.

Applicants traverse the rejections. OC001 is identical to human neurotrimin. The Examiner is directed to the attached alignments between OC001 (SEQ ID NOs. 3 and 4) and human neurotrimin. Each polypeptide represented by SEQ ID No. 3 and 4 is the total length clone and the mature clone of OC001, respectively. SEQ ID Nos. 3 and 4 fully correspond to human neurotrimin, excluding the signal sequence. Thus, 0C001 is human neurotrimin.

The Examiner further alleges that one skilled in the art is unable to ascertain the function and activity of OC001. However, it is established that 0C001 causes neurite outgrowth. For

example, the present specification discloses that OC001 shares activities with the nervous cell adhesion molecular family including (rat) neurotrimin and opioid-binding cell adhesion molecular (hereinafter, OBCAM). Since OC001 is 70% and 91% homologous to (rat) neurotrimin and OBCAM respectively, one of ordinary skill in the art would appreciate that OC001 shares neurotrimin and OBCAM properties based on homology alone. As of Applicants' filing date, Pimenta et al. disclosed that neurotrimin and OBCAM, together with LAMP and GP55, were IgLON family members, a subgroup of the immunoglobulin superfamily. (Enclosed herewith, *Neuron*, 1995, 15, 287-297.) Further, Gil et al. and Wilson et al. acknowledged that receptors belonging to the IgLON family induce neurite outgrowth via homophilic interactions with neuronal cells. The same reference discloses that inhibition occurs, via heterophilic interactions with neuronal cells, potentially, in the absence of the receptor.

Furthermore, Gil et al. disclose that neurotrimin induces neurite outgrowth in DRG neurons whereas neurotrimin inhibits outgrowth in SRG neurons, and that said properties are comparable to homophilic interactions with DRG neurons in which neurotrimin is expressed and to heterophilic interactions with SRG neurons in which neurotrimin is not expressed, respectively.

Wilson et al. disclose that GP55 inhibits the neurite outgrowth of DRG neurons. However, Gil et al. disclose, second paragraph, right column, page 9322, "Interestingly, the effects of gp55 on outgrowth were observed with chick E9 DRG neurons, which have not yet begun to express these proteins, indicating a heterophilic mechanism of inhibition (Clarke and Moss, *European Journal of Neuroscience*, 1997, 9, 334-241). It is not yet known whether these chick IgLON members will promote the outgrowth of older DRG neurons that express gp55."

More specifically, Gil et al. disclose that GP55 is not expressed in the DRG neurons used by Wilson et al. and thus would induce the neurite outgrowth in older DRG neurons in which GP55 is expressed. Further, Zhukareva and Levitt (*Development*, 1995, 121(4), 1161-1172, attached herewith) disclose that LAMP is a homophilic cell adhesion molecule which will stimulate outgrowth of neurons which express LAMP but appears to neither stimulate nor inhibit the growth of LAMP negative neurons. Gil et al. and Wilson et al. acknowledge that IgLON receptors at least function to induce the neurite outgrowth of neuronal cells depending on the recipient.

The Examiner asserts that the function of OC001 is unknown because of its dual functionality. However, it is disclosed that since the dual aspect of OC001 is based on a recipient and not on OC001 proper, OC001 regulates neurite outgrowth of neuronal cells vis-à-vis the recipient.

The reference upon which the Examiner relies, namely McNamee, was disclosed in 2002 and does not reflect the appropriate state of the art. Curiously, only McNamee et al. allege that any receptor belonging to the IgLON family does not relate to the neurite outgrowth.

In view of the foregoing, Applicants submit that the claimed OC001 is supported by both a specific and substantial asserted utility and a well-established utility; therefore, reconsideration and withdrawal of the § 101 and § 112, first paragraph, rejections are respectfully requested.

III. The Claims Are Adequately Described Under 35 U.S.C. § 112, First Paragraph

At page 12 and page 14 of the Office Action, the Office rejects claims 1 and 10 under 35 U.S.C. § 112, first paragraph as allegedly not being supported by a written description and as containing new matter, respectively.

Applicants herewith amend claims 1 and 10 without prejudice or disclaimer. Applicants' amendments overcome the rejections.

Withdrawal of the rejections is therefore kindly requested.

IV. The Claims are Definite Under 35 U.S.C. § 112, Second Paragraph

In the Office Action at page 15, paragraph 12, the Office rejects claims 1, 2 and 10 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

Applicants herewith amend claims 1 and 10, without prejudice or disclaimer, and cancel claim 2. Applicants' amendments overcome the rejection.

Withdrawal of the rejection is therefore requested.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/W. Simmons/

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WASHINGTON DC SUGHRUE/265550

65565
CUSTOMER NUMBER

Date: September 11, 2007

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ClustalW Result                                          2007-07-24(Tue) 15:36:50
CLUSTAL W (1.83) Multiple Sequence Alignments

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Sequence 2: human_neurotrimin      344 aa
Sequence 3: SEQ_ID_NO_4            313 aa
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Sequences (1:3) Aligned. Score: 100
Sequences (2:2) Aligned. Score: 100
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Sequences (3:2) Aligned. Score: 100
Sequences (3:3) Aligned. Score: 100
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Start of Multiple Alignment
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Aligning...
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Group 2: Sequences:   3      Score:7150
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CLUSTAL W (1.83) multiple sequence alignment

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SEQ_ID_NO_3            VSEDEYLEIQGITREQSGDYECSASNDVAAPVVRRVKVTVNYPPYISEAKGTGVPVGQKG
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human_neurotrimin      KLGHTNASIMLFGPGAVSEVSNGTSRRAGCVWLLPLLVLHLLLKF
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SEQ_ID_NO_3            KLGHTNASIMLFGPGAVSEVSNGTSRRAGCVWLLPLLVLHLLLKF
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The limbic system-associated membrane protein (LAMP) selectively mediates interactions with specific central neuron populations

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SUMMARY

The limbic system-associated membrane protein (LAMP) is a 64-68×10^3 M_r glycoprotein that is expressed by subsets of neurons that are functionally interconnected. LAMP exhibits characteristics that are indicative of a developmentally significant protein, such as an early and restricted pattern of expression and the ability to mediate specific fiber-target interactions. A potential, selective adhesive mechanism by which LAMP may regulate the formation of specific circuits is investigated in the present experiments. LAMP is readily released from intact membranes by phosphatidyl inositol-specific phospholipase C. Purified, native LAMP, isolated by PI-PLC digestion and immunoaffinity chromatography, is capable of mediating fluorescent Covasphere aggregation via homophilic binding. To test the ability of LAMP to selectively facilitate substrate adhesion and growth of neurons from LAMP-positive, in contrast to LAMP-negative regions of the developing brain, purified LAMP was dotted onto nitrocellulose-coated dishes and test cells plated. Limbic neurons from perirhinal cortex bind specifically to substrate-bound LAMP within 4 hours, forming small cell aggregates with short neuritic processes that continue to grow through a 48 hour period of monitoring. Preincubation of cells with anti-LAMP has a modest effect on cell binding but significantly reduces initiation of process growth. Non-limbic neurons from somatosensory cortex and olfactory bulb fail to bind or extend processes on the LAMP substrate to any significant extent. All cell populations bind equally well and form neurites on poly-D-lysine and laminin. The present results provide direct evidence that LAMP can specifically facilitate interactions with select neurons in the CNS during development. The data support the concept that patterned expression of unique cell adhesion molecules in functionally related regions of the mammalian brain can regulate circuit formation.

Key words: neural development, cell surface, neurite outgrowth, phosphatidylinositol-specific phospholipase C, chemospecificity

INTRODUCTION

Cell adhesion molecules underlie the molecular mechanisms that mediate cell migration, cell-cell and cell-matrix adhesion, cytodifferentiation and synaptogenesis during development of the central nervous system (CNS; Goodman et al., 1984; Dodd and Jessell, 1988; Schachner, 1991; Edelman and Crossin, 1991; Stappert and Kemler, 1993). Adhesive interactions between surfaces are particularly critical during development for regulating cell aggregation (Huang et al., 1993), neurite outgrowth (Lemmon et al., 1989; Rathjen et al., 1991; Norenberg et al., 1992), neuron-glia interactions (Antonicek et al., 1987; Grumet and Edelman, 1988; Smith et al., 1993) and specific pathfinding (Goodman and Shatz, 1993). Several families of cell adhesion molecules (CAMs) have been identified, including the integrins (Reichart and Tomaselli, 1991; Hynes, 1992), Ca^{2+}- dependent cadherins (Takeishi, 1991) and the Ig superfamily (Cunningham et al., 1987; Grumet, 1992). In vertebrates, members of the Ig-superfamily exist as transmembrane and phosphatidyl inositol (GPI) linked proteins that are concentrated on axonal surfaces during embryonic development (Ferguson and Williams, 1988; Low and Saltiel, 1988; Rosen et al., 1992), implicating them in fiber growth (Rathjen, 1991; Brumendorf et al., 1993; Huang et al., 1993). Some proteins, such as NCAM and L1, are present on most axons (Moos et al., 1988; Rutishauer et al., 1988; Doherty et al., 1990), whereas others, such as TAG-1 (Dodd et al., 1988; Karagogeos et al., 1991), Ng-CAM (Grumet, 1992; Krushel et al., 1993), DM-GRASP/SC-1 (Tanaka et al., 1991; Burns et al., 1991; A. DeBernardo and S. Chang, unpublished observation) and connectin (Nose et al., 1992) are expressed in a more restricted pattern on subsets of axons at certain developmental stages.

The widely distributed CAMs (Chang et al., 1987; Lagenaur and Lemmon, 1987; Doherty et al., 1991; Edelman and Crossin, 1991; Kuhn et al., 1991; Zuellig et al., 1992; Rader et al., 1993) can mediate neurite outgrowth of a wide variety of cells. Adhesion proteins in invertebrates that are expressed in

a restricted fashion, including the invertebrate fasciclins (Bastiani et al., 1987; Harrelson and Goodman, 1988; Elkins et al., 1990; Kolodkin et al., 1992; Nose et al., 1994) and other proteins (Krantz and Zipursky, 1989; Wang et al., 1992; Bastiani et al., 1992; Singer et al., 1992), appear to selectively mediate adhesion and pathfinding. Fewer candidate molecules in the vertebrate brain have been investigated. In mammals, the limbic-system associated membrane protein (LAMP), a 64-68×10^3 M_r glycoprotein (Zacco et al., 1990), exhibits a specific pattern of expression in cortical and subcortical limbic areas, which are related through specific circuits that mediate cognitive behavior, memory and learning and autonomic functions (Levitt, 1984; Levitt et al., 1986; Zacco et al. 1990). The specific distribution of LAMP is seen initially early in fetal development (Horton and Levitt, 1988; Ferri and Levitt, 1993). Subsets of fetal neurons are LAMP immunoreactive on their soma, dendrites and axons. Immunoreactive protein eventually is lost on axons postnatally in the rat as synapse formation occurs, but is maintained in the dendritic and somal compartments of mature limbic neurons (Zacco et al., 1990). Recent antibody blocking experiments have revealed that LAMP is a critical element necessary for normal circuit formation in the limbic system, including the septo-hippocampal connection (Keller et al., 1989) and hippocampal mossy fibers (Barbe and Levitt, 1992; Pimenta, A.F., Zhukareva, V.A., Barbe, M.F., Reinoso, B., Grimley, C., Henzel, B., Fisher, I., Levitt, P., unpublished observations). The mechanism underlying specific mediation of limbic connectivity is unknown. In an effort to understand more of the molecular features of LAMP and to define physiological characteristics that could subserve the important developmental functions in which LAMP participates, several different biochemical and cell biological experiments were undertaken. The results indicate that LAMP is a GPI-linked membrane protein, and most important, it facilitates adhesive interactions in a selective manner among specific groups of developing limbic neurons.

MATERIALS AND METHODS

Adult female Sprague-Dawley albino rats (Holtzman Farm) were housed in a 12 hours light:dark cycle with free access to food and water. Timed-pregnant rats were obtained from the same supplier.

Release of LAMP by PI-PLC

Adult rat hippocampal membranes were used for the characterization and isolation of LAMP. The membranes were isolated as described previously using a sucrose gradient method (Zacco et al., 1990) and treated with GPI-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. The PI-PLC enzymes were gifts from Dr M. Low (Columbia University), Dr I. Caras (Genentech, Inc.) and Dr J. Volwerk (University of Oregon). The membranes were incubated with 3 Units/5 mg protein for 2 hours at 37°C with vigorous shaking. To facilitate access of the enzyme to material sequestered in spontaneously sealed membrane vesicles that form after homogenization, two freeze-thaw cycles were performed during the 2 hour enzyme incubation. After enzyme digestion, the membranes were pelleted and the supernatant subjected to immunoaffinity chromatography. In some experiments, hippocampal membranes, either before or after enzyme digestion, were treated with 4% CHAPS to extract LAMP, as described previously (Zacco et al., 1990). For analysis of PI-PLC released proteins, samples were run on 10% SDS gels using standard methods (Laemmli, 1970). For western blot analysis of LAMP, protein samples were diluted with modified sample buffer containing 0.2% SDS and the nitrocellulose membranes processed as described previously (Zacco et al., 1990). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham). BCA protein assay (Pierce) was used with bovine serum albumin as a standard.

PI-PLC treatment of cultured cells expressing LAMP was also performed. Primary monolayer cultures of hippocampal neurons (embryonic day 16) were prepared and grown as described previously (Qian et al., 1992) in chemically defined media (Bottenstein, 1985). We also used the SN56 immortalized cell line (kindly provided by Dr B. Wainer, Albert Einstein College of Medicine; Hammond et al., 1990; Lee et al., 1990). We determined previously that SN56 cells constitutively express LAMP on their cell surface (B. Miller and P. Levitt, unpublished observations). Both primary cells and SN56 cells were incubated with PI-PLC for 1 hour at 37°C, washed extensively with complete medium and then immunostained with anti-LAMP. Live cultures were incubated for 1 hour at 37°C with 1:50 dilution of anti-LAMP, followed by incubation with a 1:50 dilution of fluorescein-conjugated goat anti-mouse IgG (Jackson Immunoreagents), and then brief fixation in 10% formalin before mounting in PBS/glycerol.

Biosynthetic radiolabeling and immunoprecipitation of LAMP

Monolayers of SN56 cells in 100 mm dishes (5×10^6 cells) were grown in DMEM, 10% FCS and radiolabeled 48 hours after initial plating with [^{35}S]methionine (1400 Ci/mM, 50 μCi, NEN/DuPont) in methionine-free MEM (Sigma). Following extensive washing with PBS, cells were gently suspended in 0.5 ml Hank's Balanced Salt Solution and treated for 1 hour at 37°C with PI-PLC, 1U/ml cell suspension. Cells were centrifuged at 1000 *g*, and the supernatant recentrifuged at 14,000 *g* to ensure complete sedimentation of cellular debris followed by immunoprecipitation with anti-LAMP coupled to Protein A-Sepharose (Pharmacia). Samples were subjected to 10% SDS gel electrophoresis, then the gel was exposed to autoradiography Enhancer (DuPont), dried, and radiolabeled proteins visualized by exposure to Kodak XAR 5 film.

Covasphere aggregation

Fluorescent beads (Covaspheres; 0.5 μm diameter; Duke Scientific) were used to assay the ability of LAMP to mediate aggregation of the normally monomeric beads. Ten μl bead solution was incubated for 1 hour at room temperature with 10 μl CHAPS-solubilized, immunoaffinity-purified LAMP (10 μg/ml). Covaspheres were then washed and resuspended in PBS/5% BSA. Controls included beads coated with bovine serum albumin. Covasphere pretreatment with Fab′ fragments of anti-LAMP or Fab′ fragments of anti-fibrinogen was performed for 30 minutes at room temperature and unbound antibody was removed by centrifugation of particles for 10 minutes and washing in PBS/5% BSA. The beads were sonicated (10-30 seconds) to disrupt any aggregation that might have occurred during protein incubation before assessing specific aggregation. After sonication, 5 μl of the protein-coated beads were added to 1 ml of PBS. Aggregation was monitored visually on a fluorescence microscope and photographed, or analyzed by a Fluorescence-Activated Cell Sorter (FACS). The FACS analysis was performed after 15 minutes of incubation and used to determine changes in the number of particles in solution larger than 2 μm. Complexes of 1-4 beads (<2 μm) were considered single, non-aggregated particles.

Cell adhesion assay

The nitrocellulose adhesion assay of Lagenaur and Lemmon (1987) was used. At least 6 independent experiments, each run in duplicate, were performed for each condition and with each population of fetal brain cells. Test protein samples were applied to the dried nitrocellulose film in 4 μl droplets to an area that was marked by a 3 mm^2 template on the underside of the culture dish. Poly-D-lysine (Sigma;

0.1 mg/ml), laminin (Gibco; 10 μg/ml) and PI-PLC-released, affinity purified LAMP (50 μg/ml) were spotted in the same dish so that a single cell suspension could be tested simultaneously against each substrate. In experiments using anti-LAMP to block the dotted LAMP substrate, additional binding sites on the nitrocellulose first were blocked by incubation for 30 minutes in DMEM/10% FCS. The plate was washed and the anti-LAMP (100 μg/ml) was dotted on the already adsorbed LAMP, allowed to bind for 1 hour at room temperature and washed with DMEM. In some experiments, anti-LAMP was dotted onto nitrocellulose as a substrate, and then blocked and washed. Monolayer cultures of E16 olfactory bulb, somatosensory cortex and perirhinal cortex were prepared. Cortical areas were dissected according to recently developed methods (Ferri and Levitt, 1993) and all tissue was subjected to collagenase/dispase (Worthington Biochem.Co.) digestion prior to trituration with a fine bore pipette. Cells were plated at a density of $5\times10^4/cm^2$ in DMEM/10% FCS medium. In all experiments, cells did not bind to areas outside of the 3 mm^2 zone that contained the test substrates. Cultures were examined and photographed at 4, 18 and 48 hours. For quantitative analysis, the number of cell aggregates (>5 cells) was calculated in the entire 3 mm^2 area. In addition, the diameter (shortest axis) of each aggregate was measured with an eye piece micrometer and the mean was calculated.

An additional experiment was performed in which LAMP on the cultured neurons was blocked with antibody. Cells from perirhinal cortex were allowed to recover for up to 4 hours at 37°C, followed by incubation with anti-LAMP (1:50 dilution) for 1 hour. After washing, cells were plated at a density 5×10^5 cells/ml in DMEM/10% FCS on the nitrocellulose-coated Petri dishes with LAMP or polylysine as a substrate. After 18 hours of incubation, unbound cells and cell aggregates were gently washed off and the remaining cells were analyzed.

RESULTS

LAMP is a GPI-linked protein

Previous data from this laboratory, obtained from differential centrifugation and detergent extraction studies, demonstrated that LAMP behaves as a typical membrane protein (Zacco et al., 1990). The ability of CHAPS, a detergent with a high CMC (Hooper and Turner, 1988), to solubilize LAMP led to the hypothesis that LAMP is a GPI-anchored protein. We tested this by using an enzyme preparation that specifically digests GPI membrane linkages (Low, 1989; Low and Saltiel, 1988; Low et al., 1988). Membranes isolated from the hippocampus and treated with PI-PLC released substantial LAMP immunoreactivity into the supernatant (Fig. 1A). This PI-PLC release could be blocked, though incompletely, with the addition of $ZnCl_2$, a known inhibitor of PI-PLC activity (Taguchi et al., 1980). To estimate the relative amount of LAMP that exhibited the GPI-linkage, hippocampal cell membranes, that had been treated enzymatically, were extracted subsequently with 4% CHAPS under conditions that normally solubilize substantial amounts of LAMP. No additional LAMP was removed from the membranes with the detergent (Fig. 1A), indicating all of the protein is GPI-linked. SN56 cells were subjected to metabolic labeling with [^{35}S]methionine, treated with PI-PLC to release LAMP and the liberated protein in the supernatant was immunoprecipitated (Fig. 1B); control cultures were treated with buffer alone. Only supernatants from PI-PLC-treated cultures contained detectable LAMP, with the band running between $64\text{-}68\times10^3$ M_r. We failed to immunoprecipitate radiolabeled LAMP from the supernatants of control cultures, indicating that LAMP is not released constitutively from the cultured cell line.

Live limbic cortical neurons and SN56 cells were stained in culture with anti-LAMP either before or after PI-PLC treatment (Fig. 2). LAMP was visible as punctate immunoreactivity on the surfaces of somata and neurites of untreated cultures (Fig. 2A,E). The staining in both cell population was completely eliminated with PI-PLC incubation (Fig. 2B,F), but was unaffected by exposure to nonspecific PLC, an enzyme that hydrolyzes phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, but not phosphatidylinositol lipids. We found, however, that the non-specific PLC additionally resulted in cytoplasmic localization of anti-LAMP in the live, cultured cells (Fig. 2C,G). To determine whether the non-specific PLC effectively permeabilized cell membranes, enzyme-treated, live primary neurons were incubated with an antibody against a specific cytoplasmic cytoskeletal protein, microtubule associated protein 2 (MAP2) (Crandall and Fischer, 1989). Anti-MAP2 gained direct access to the cytoplasmic compartment without any other treatments (Fig. 2D), suggesting a similar route in the live cells for anti-LAMP.

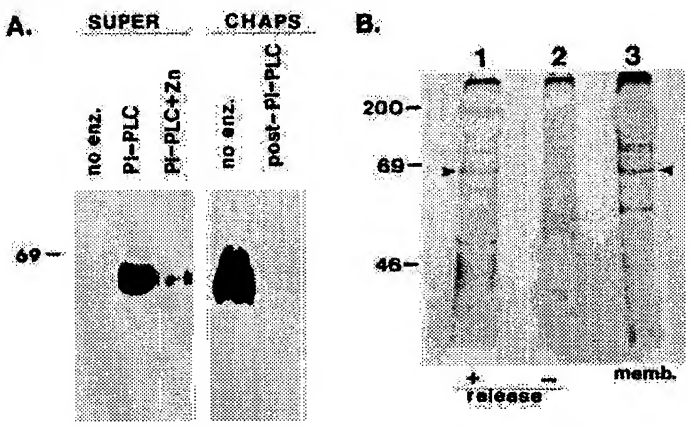

Fig. 1. (A) Western blot analysis of LAMP redistribution after PI-PLC treatment of hippocampal membranes. SUPER indicates samples collected from supernatants after treatment of the membranes. In control experiments, no LAMP is detected in the high speed supernatant without enzyme treatment (no enz.). Treatment of the membranes with PI-PLC for 2 hours at 37°C releases a large amount of LAMP into the supernatant (PI-PLC). In the presence of 5 mM $ZnCl_2$, PI-PLC releases a much smaller amount of LAMP. *CHAPS* denotes samples after detergent extraction. Membranes solubilized with 4% CHAPS for 1 hour at 4°C, without prior enzyme treatment, release a large amount of LAMP (no enz.). In contrast, treatment of membranes with CHAPS after PI-PLC digestion fails to solubilize additional, immunodetectible LAMP (post-PI-PLC). The position of the protein marker is shown on the left.
(B) Autoradiograms show [^{35}S]methionine incorporation into LAMP after metabolic labeling of SN56 cells, followed by immunoprecipitation with mouse anti-LAMP. Labeled LAMP, running as a band between $64\text{-}68\times10^3$ M_r, released into the incubation medium after cleavage with PI-PLC (lane 1, '+'). Without enzyme treatment, no detectable radioactivity is found in the medium (lane 2, '–'). LAMP labeled with [^{35}S]methionine also is present in the CHAPS-solubilized fraction from cell membranes without PI-PLC treatment (lane 3, 'memb.'). Arrowheads denote the LAMP band. The position of protein markers is shown on the left.

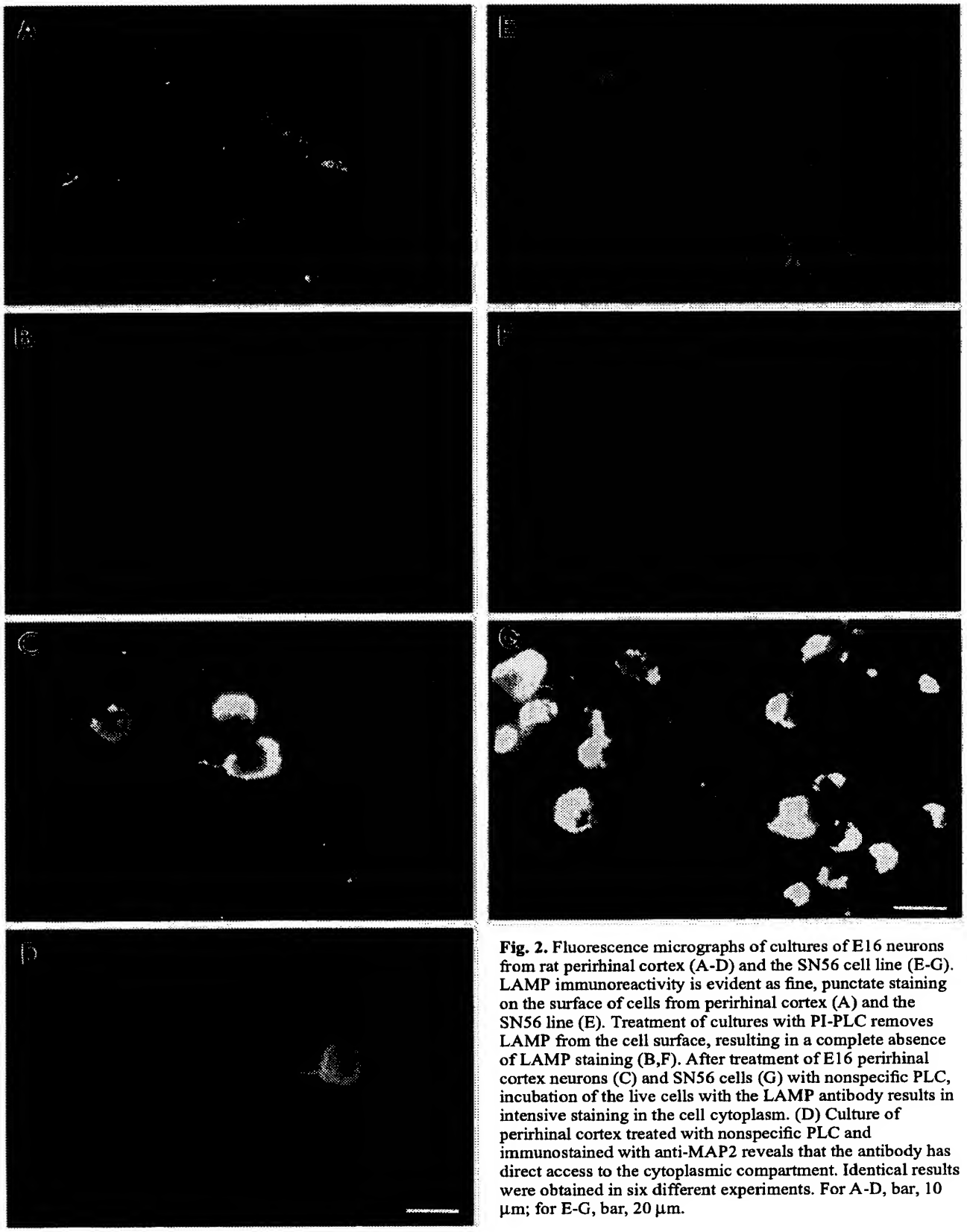

Fig. 2. Fluorescence micrographs of cultures of E16 neurons from rat perirhinal cortex (A-D) and the SN56 cell line (E-G). LAMP immunoreactivity is evident as fine, punctate staining on the surface of cells from perirhinal cortex (A) and the SN56 line (E). Treatment of cultures with PI-PLC removes LAMP from the cell surface, resulting in a complete absence of LAMP staining (B,F). After treatment of E16 perirhinal cortex neurons (C) and SN56 cells (G) with nonspecific PLC, incubation of the live cells with the LAMP antibody results in intensive staining in the cell cytoplasm. (D) Culture of perirhinal cortex treated with nonspecific PLC and immunostained with anti-MAP2 reveals that the antibody has direct access to the cytoplasmic compartment. Identical results were obtained in six different experiments. For A-D, bar, 10 μm; for E-G, bar, 20 μm.

LAMP mediates homophilic binding

Given previous antibody perturbation studies that documented the role of LAMP in afferent targeting (Keller et al., 1989; Barbe and Levitt, 1992; Pimenta, A.F., Zhukareva, V.A., Reinoso, B., Grimley, C., Henzel, B., Fisher, I., Levitt, P., unpublished observations), we investigated potential mechanisms by which the protein could exert its influence. First, PI-PLC-released, affinity-purified LAMP from the hippocampus was immobilized on fluorescent beads (Covaspheres) to assess the ability of the protein to mediate specific aggregation of the normally monomeric beads. LAMP-coated Covaspheres rapidly aggregated within 10-30 minutes (Fig. 3A), suggesting the possibility of a homophilic interaction between LAMP molecules that is sufficient to mediate adhesion. Bead aggregation was blocked when the incubations occurred in the presence of Fab′ fragments of anti-LAMP (Fig. 3B). BSA-coated Covaspheres failed to aggregate, even when incubated for up to 30 minutes (Fig. 3C). The number of aggregates of beads coated with LAMP (particles >2 μm) that formed in solution was assessed by FACS analysis. More than 30% of the particles formed multimeric complexes after 15 minutes of incubation. The aggregation was blocked by addition of Fab′ fragments of anti-LAMP, but not by incubation with anti-fibrinogen (Fig. 3D), which blocks fibrinogen-mediated aggregation (data not shown).

LAMP facilitates limbic neuron adhesion and outgrowth

Direct evidence for LAMP as a molecule that promotes cell adhesion and neurite outgrowth was obtained using a nitrocellulose-coated substrate assay. We tested 3 neuronal populations for their ability to adhere to LAMP, laminin (data not shown) and poly-D-lysine. Olfactory bulb and somatosensory cortex are two regions that contain mostly LAMP-negative cells (Levitt, 1984; Horton and Levitt, 1988; Ferri and Levitt, 1993); perirhinal cortex contains mostly LAMP-positive cells beginning at E15 and continuing throughout development. Cells from all three regions bound to poly-D-lysine and extended neurites (Figs 4A-C, 5A,C). In general, many small aggregates formed with neuritic processes extending between the groups of cells. Isolated cells also were present and exhibited modest, but obvious, neurite outgrowth during the 4-48 hours period. Cells from all 3 regions grew similarly on

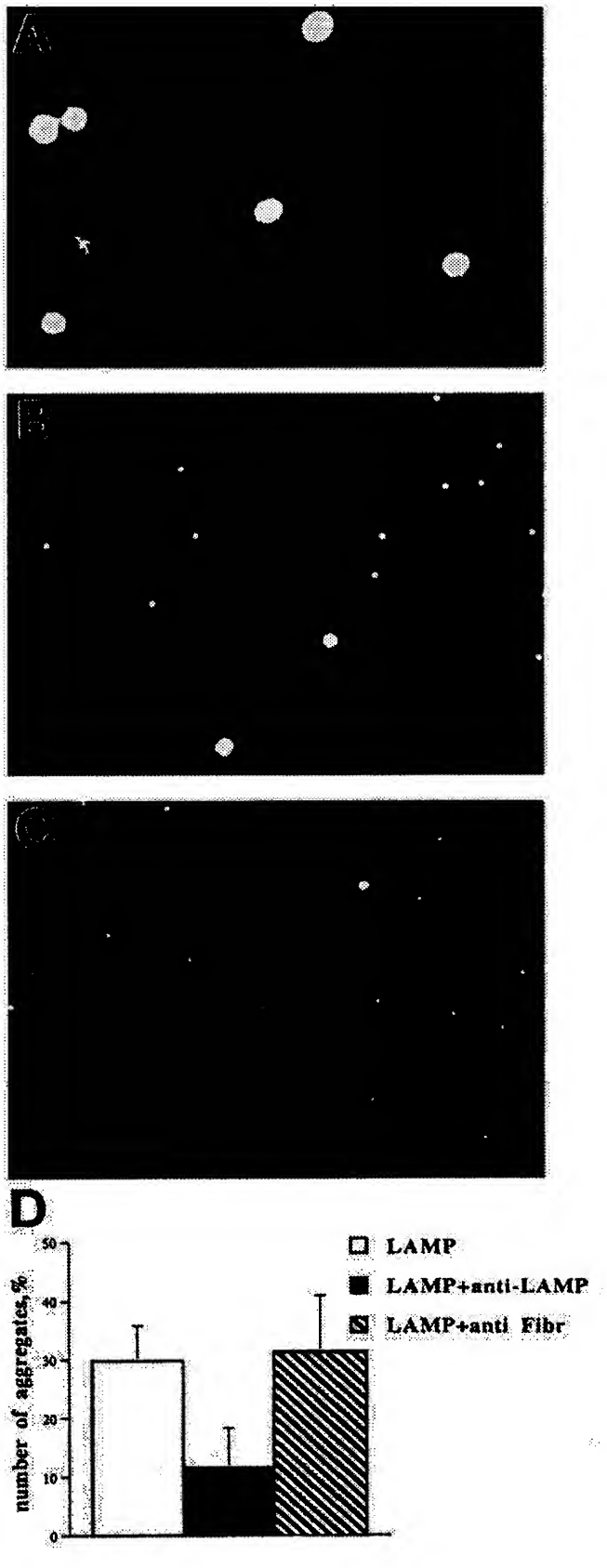

Fig. 3. Fluorescence micrographs depicting analysis of LAMP-mediated homophilic binding. (A) Covaspheres coated with affinity-purified LAMP undergo rapid aggregation during the 15 minute incubation period. (B) The aggregation is blocked by incubation with anti-LAMP Fab′ fragments. (C) As a control for nonspecific binding, beads coated with bovine serum albumin fail to aggregate. Bar, 10 μm for all micrographs. (D) Quantitative FACS analysis of aggregation of LAMP-coated Covaspheres alone (open bar), incubated with Fab′ fragments of anti-LAMP (solid bar), or Fab′ fragments of anti-fibrinogen (hatched bar). There is specific reduction in aggregate number with anti-LAMP, whereas the anti-fibrinogen has no effect on the formation of aggregates by the LAMP-coated beads. The data are expressed as the percentage of the total particles in solution whose diameter is greater than 2 μm, which was considered an aggregate. Each monomeric bead is approximately 0.5 μm.

laminin, with fewer cell aggregates binding but exhibiting longer neurites (data not show).

Cells from perirhinal cortex adhered to the LAMP substrate in a time-dependent manner, with numerous aggregates and few single cells binding to the surface (Fig. 4D-F). In sharp contrast, cells from non-limbic areas exhibited very weak

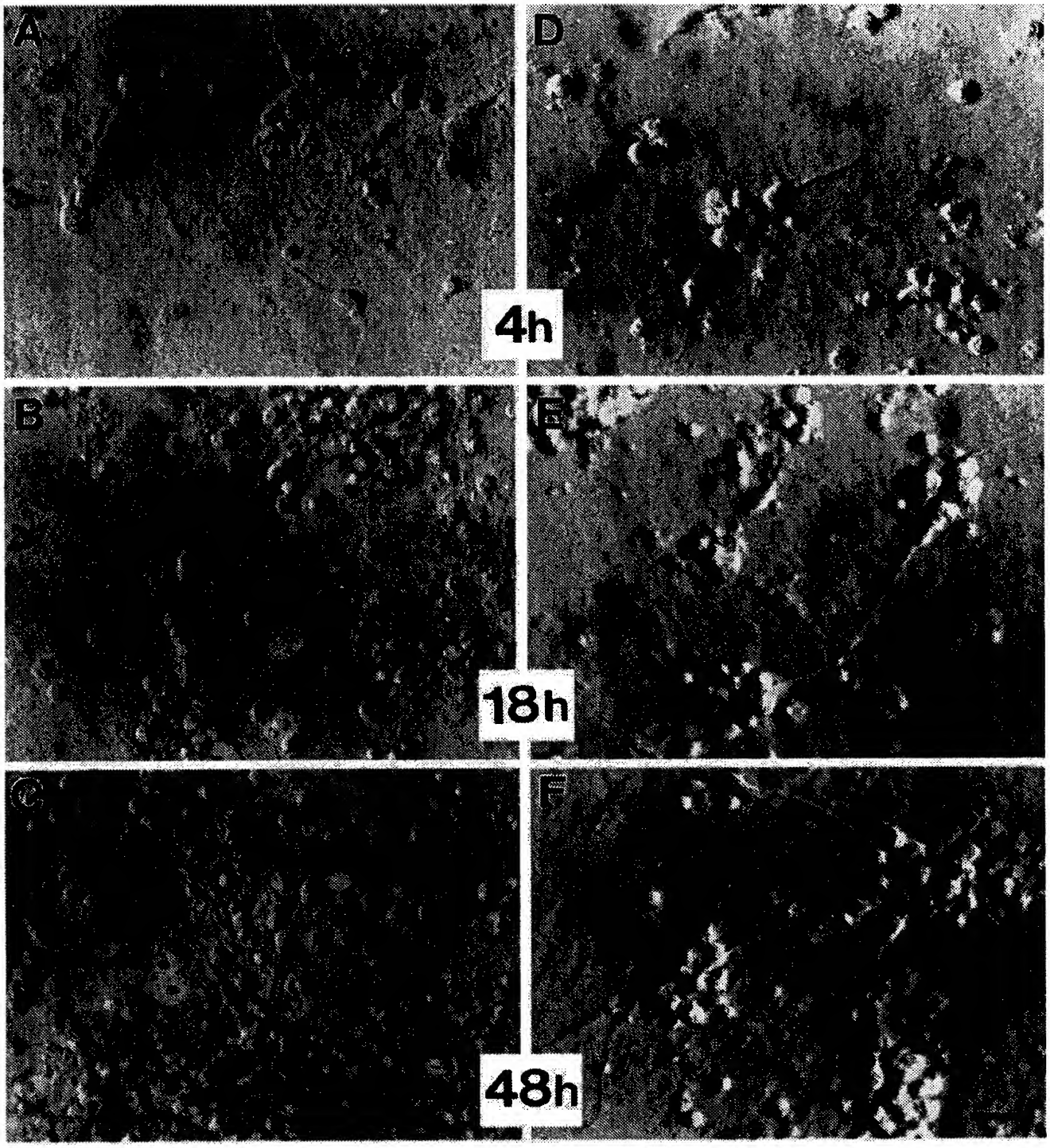

Fig. 4. PI-PLC liberated LAMP serves as a specific adhesive substrate for limbic neurons in primary culture. Bright-field photomicrographs show the growth of the cells isolated from E16 perirhinal cortex on poly-D-lysine (A-C) or PI-PLC released LAMP (D-F). The pairs at each time point were from the same dish. (A,D) At 4 hours, cells bind and extend short processes on both PDL and LAMP. (B,E) At 18 hours, cell aggregates and long neurites are evident on both substrates. (C,F) At 48 hours, more extensive aggregation and outgrowth is evident on both substrates. Bar, 20 μm.

binding to the LAMP substrate; only an occasional cell from the olfactory bulb bound (Fig. 5B), while somewhat more cells from somatosensory cortex exhibited binding to LAMP (Fig. 5D). We never observed any aggregates when cells from either non-limbic region were plated onto the LAMP substrate. Of those cells from somatosensory and olfactory bulb that bound, very few extended neurites. In contrast, neurites extending from perirhinal cells on the LAMP substrate were always evident, even after only 4 hours in culture. Cells harvested from younger (E15) or older (E18) embryos showed the same binding characteristics on LAMP (data not shown).

LAMP-LAMP interactions mediate limbic neuron binding

Specific interactions between substrate-bound LAMP and limbic neurons that are known to express LAMP were evident in the culture studies. To determine whether this binding could be mediated through homophilic interactions (Murray and Jenssen, 1992), we performed several antibody blocking experiments. As expected, pretreatment of the culture dish with anti-LAMP reduced cell adhesion and fiber outgrowth of perirhinal cells only on the LAMP substrate (Fig. 6A), having no effect on neurite outgrowth on poly-D-lysine (Fig. 6B) or laminin

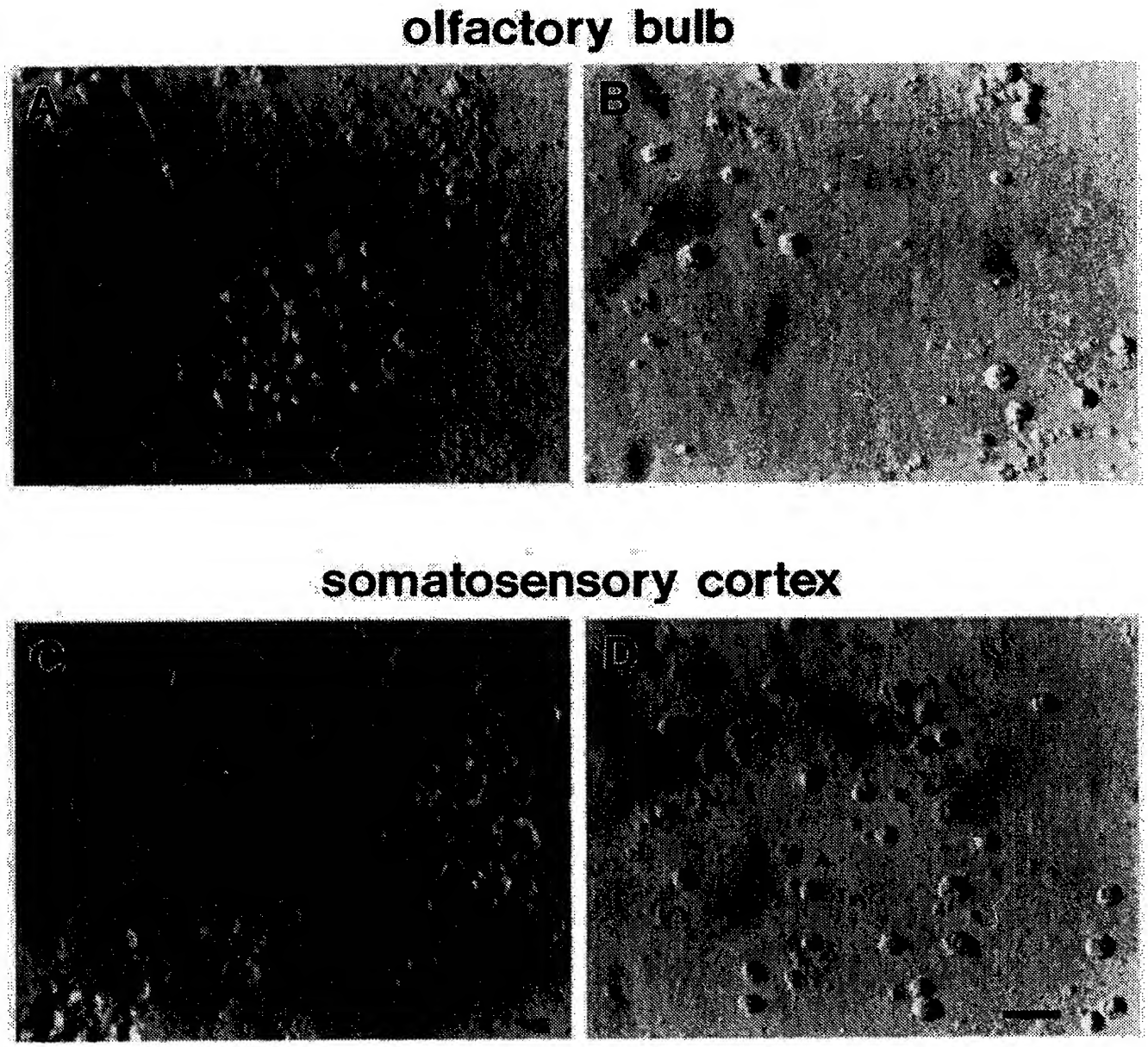

Fig. 5. Non-limbic neurons in primary culture do not adhere or grow on a LAMP substrate. Bright-field photomicrographs illustrate growth, after 18 hours in culture, of cells from E16 olfactory bulb and somatosensory cortex on poly-D-lysine (A,C) or affinity-pure LAMP (B,D). Both cell populations adhere mostly as aggregates and extend processes on the PDL. In the same dish, cells over the LAMP spots exhibit poor binding, with relatively few cells remaining after gentle washing. Of those cells that bind, none extend neurites. Bar, 20 μm.

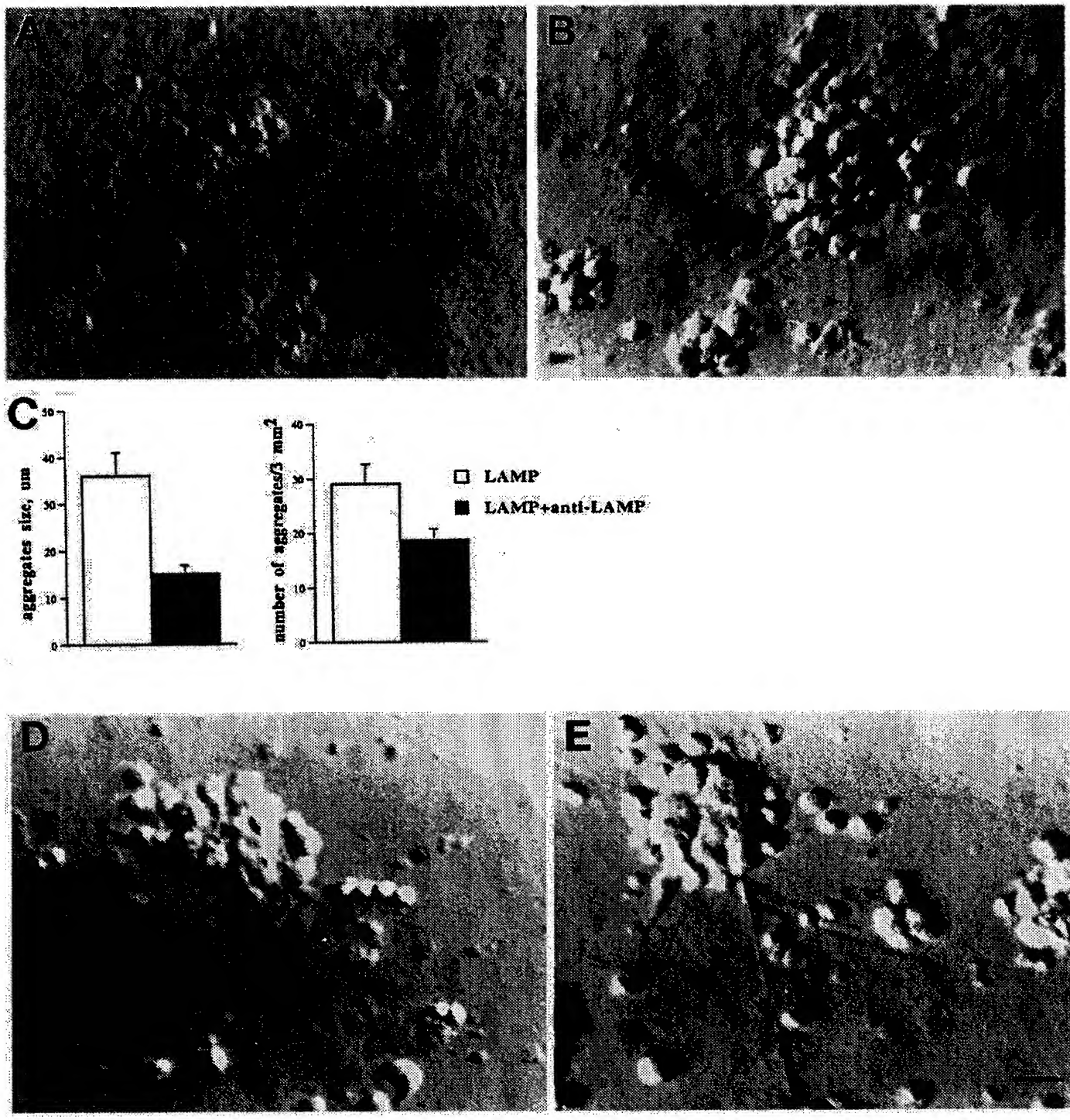

Fig. 6. Cell binding to LAMP acts by homophilic interactions. (A) Pretreatment of the LAMP-coated substrate with anti-LAMP blocks E16 perirhinal cell binding and aggregation at 18 hours. (B) Pretreatment of the PDL substrate with anti-LAMP has no effect on cell binding or outgrowth. (C) The number and size of cell aggregates formed after plating on nitrocellulose-coated dishes dotted with LAMP in the presence or absence of anti-LAMP was determined. Diameter of aggregates (the shortest axis) is reduced more than 2-fold by the antibody treatment, while the number of aggregates with more than 5 cells is decreased about 1.5 fold. (D) Photomicrograph of E16 perirhinal cells that were pre-treated for 4 hours with anti-LAMP prior to plating on a LAMP substrate. Here, a culture is depicted in which cell aggregates bound, but failed to develop any neurites on the LAMP-substrate. (E) Non-treated cells from the same tissue dissection show typical aggregation and neurite extension (arrowhead) on the LAMP substrate. Bar, 20 μm for all micrographs.

(data not shown). Anti-LAMP treatment reduced the number of aggregates formed by the perirhinal cells (Fig. 6C). When perirhinal cells were preincubated with anti-LAMP for 1 hour immediately after tissue dissociation, they were still able to bind to the LAMP substrate and extend processes (data not shown). This may reflect the absence of membrane-bound LAMP soon after the dissociation process. To test this possibility, after trituration cells were allowed to recover in suspension for 4 hours at 37°C and then preincubated with anti-LAMP before being plated on the substrate. Untreated cells formed typical aggregates and extended processes on the LAMP substrate, even after 4 hours (data not shown) and throughout the culture period (Fig. 6D). Cells treated with anti-LAMP formed aggregates after plating on the LAMP-substrate, but were easily removed after gentle washing; those cells that adhered to the substrate failed to extend any processes after 18 hours in culture (Fig. 6E). Treatment of the cell suspension with anti-LAMP did not alter cell binding and neurite outgrowth on poly-D-lysine (data not shown).

DISCUSSION

There is now substantial evidence that cell adhesion molecules are particularly important in mediating cell migration, neurite outgrowth and pathfinding. The role of CAMs in directing specific groups of axons to appropriate targets has become clearer from studies in invertebrates, where, for example, mis-expression studies reveal important contributions to axon targeting by specific molecules (Lin and Goodman, 1994; Nose et al., 1994). LAMP exhibits characteristics of a protein that can regulate specific cell-cell interactions, such as its early and restricted pattern of expression and the disruption of fiber-target interactions by antibodies against the LAMP protein (Keller et al., 1989; Barbe and Levitt, 1992; Pimenta, A.F., Zhukareva, V.A., Barbe, M.F., Reinoso, B., Grimley, C., Henzel, B., Fischer, I., Levitt, P., unpublished observations). The results presented here demonstrate that LAMP can mediate specific adhesive interactions with populations of limbic neurons, but fails to promote the adhesion of non-limbic cells.

LAMP is a GPI-anchored protein

It has been suggested that membrane proteins that are soluble in CHAPS are likely to have a GPI anchor (Hooper and Turner, 1988). The present results directly demonstrate that LAMP has a GPI membrane linkage, shown on isolated membranes from the adult hippocampus and on intact, cultured cells. In fact, post-enzymatic extraction of the membranes with CHAPS failed to solubilize detectable LAMP immunoreactivity. This indicates that little, if any, LAMP exists in a transmembrane form. Recent sequence analysis of LAMP cDNA clones also failed to reveal a transmembrane form (Pimenta et al., 1993; Pimenta, A.F., Zhukareva, V.A., Reinoso, B., Grimley, C., Henzel, B., Fischer, I., Levitt, P., unpublished observations). The present data from cultured cells also indicate that the major form of LAMP is likely one with the GPI-anchor. Digestion of LAMP with PI-PLC on live cells liberated a sufficient amount to visualize the metabolically labeled protein. In addition, we failed to visualize any surface immunoreactivity on individual cells and processes after the enzyme digestion. While these methods of analysis clearly demonstrate the presence of a GPI anchor, we failed to obtain reproducible results using the strategy of [^{3}H]ethanolamine metabolic labeling. We usually obtained very low, though specific immunoprecipated product that could be seen as a faint band on X-ray film. This result is common for low abundance proteins such as LAMP (Low and Saltiel, 1988).

Our control experiments, in which non-specific PLC was used to treat the cultured cells, resulted in surprising membrane permeability. This effect facilitated the entry of anti-LAMP and anti-MAP2 into the cytoplasmic compartment of live cells. The anti-LAMP localization likely represents non-specific accumulation of antibody, rather than detection of a large, soluble pool of the protein, particularly in light of our previous cell fractionation analysis in which LAMP immunoreactivity was found only in the membrane component (Zacco et al., 1990).

Developmental significance of GPI-linked proteins

GPI-linked forms of membrane proteins exhibit dynamic developmental regulation. Distinct patterns of fasciclin I expression during development may be due to variations in the cleavage of its GPI form (Hortsh and Goodman, 1990). TAG-1 also exhibits regulated patterns of expression during embryogenesis (Karagogeos et al., 1991; Felsinfeld et al., 1994). In vitro studies with purified GPI-anchored proteins directly demonstrate their ability to mediate fiber growth (Stahl et al., 1990; Chang et al., 1992). The relative advantages of utilizing GPI-linked proteins for mediating dynamic developmental interactions have been hypothesized, but remain untested. The GPI anchor could provide rapid regulation of cellular adhesion in a novel way by readily removing CAMs into the extracellular space, thus serving as a substrate for PI-specific phospholipase (Low, 1989; Rahman et al., 1992). Proteins released from the cell surface might bind to their appropriate receptor on neighboring cells to promote inhibition of cell-cell interactions (Ferguson and Williams, 1988; Furley et al., 1990). Alternatively, the extracellular GPI anchor might serve as a target for endogenous PI-specific PLC (Fox et al., 1987; Ting and Pagano, 1990; Spath et al., 1991), resulting in rapid and specific generation of intracellular second messengers (Maher, 1993). The cleavage of GPI-anchored molecules produces inositol phosphates that release Ca^{2+} from intracellular stores, and diacylglycerol (PLC cleavage) or phosphatidic acid (PLD cleavage). This has led to the hypothesis that the cleavage of GPI-anchors is part of a receptor-mediated trigger (Cross, 1987). It was shown recently that GPI-linked molecules, including Thy-1, are associated with a cytoplasmic tyrosine kinase (Stefanova et al., 1991), indicating that GPI-linked membrane constituents can indirectly interact with proteins on the inner face of the plasma membrane.

There also have been suggestions that the GPI anchor conveys more lateral mobility in the plane of the membrane than a transmembrane domain (Noda et al., 1987). As such, GPI-linked proteins would be attractive candidates for mediating the dynamic remodeling of membranes that occurs during neurite outgrowth, synaptogenesis and myelinization by allowing for transient, though specific, adhesion between the membranes of apposed cells (Chang et al., 1992; Drake et al., 1992; Rosen et al., 1992). This would facilitate surface interactions via GPI-linked adhesion molecules, yet allow the cells to retain the ability to move relative to each other.

LAMP as a mediator of cell type-specific interactions

Our previous antibody perturbation studies strongly indicate that LAMP is involved in normal circuit formation among specific populations of neurons (Keller et al., 1989; Barbe and Levitt, 1992; Pimenta, A.F., Zhukareva, V.A., Barbe, M.F., Reinoso, B., Grimley, C., Henzel, B., Fischer, I., Levitt, P., unpublished observations). The present study provides direct evidence for the specificity of LAMP activity using two approaches. The rapid, reversible aggregation of the LAMP-coated Covaspheres indicates adhesive properties, as has been shown previously for Ng-CAM and NCAM (Grumet and Edelman, 1984) and TAG-1 (Felsinfeld et al., 1994). Such interactions in a cell-free system do not directly address the ability of LAMP to mediate adhesion under more conventional physiological conditions. We therefore utilized the system developed by Lagenaur and Lemmon (1987) to test the ability of LAMP to facilitate substrate adhesion and growth. Substrate adhesion assays in general have shown that most CAMs can facilitate attachment of many different neuronal populations, although there are clear differences in the extent and type of neurite outgrowth (Zuellig et al., 1992; Brumendorf et al., 1993). The present study has shown that LAMP may be highly selective in its promotion of cell-cell interactions, because non-limbic neurons failed to bind or extend processes to any significant extent on the substrate-bound LAMP. In contrast, neurons from perirhinal limbic cortex and hippocampus (data not shown) bind specifically to LAMP. The Covasphere data indicate that this interaction is homophilic. This inference is supported by anatomical data showing that LAMP is located on both target neuronal somata and dendrites and afferent growth cones (Zacco et al., 1990).

We attempted to test for homophilic interactions by pre-treating the fetal limbic cells with anti-LAMP prior to plating. Blocking would strongly indicate that intact LAMP on the cells is required for binding to the substrate-bound LAMP. We found no changes in binding and outgrowth when cells were plated 1 hour after the tissue dissociation procedure. This could be due to the lack of anti-LAMP binding to the cell surface, reflecting the low amount of membrane-associated LAMP on the cell 1 hour after dissociation. When we extended pre-plating recovery time to 4 hours prior to incubation with the antibody, the cells formed aggregates, some of which remained bound to the LAMP-substrate after gentle washing; the cells, however, failed to extend any neurites onto the substrate, even after 18 hours. It is likely that continual exposure to excess antibody during the period of new LAMP synthesis and membrane insertion blocked a sufficient number of sites to prevent homophilic binding required for neurite outgrowth. The fact that we obtained some aggregates that bound to the substrate after antibody treatment suggests that perhaps fewer LAMP-LAMP interactions are needed for cell binding compared to neurite extension. This idea is consistent with our recent findings with primary neurons plated onto CHO cells transfected with the full-length LAMP cDNA. Here, all neuron types bound to the transfected cells, but only the limbic cortical cells extended any processes (Pimenta, A.F., Zhukareva, V.A., Barbe, M.F., Reinoso, B., Grimley, C., Henzel, B., Fischer, I., Levitt, P., unpublished observations). It also is possible that soma binding and neurite outgrowth are mediated through different adhesive mechanisms, as recently implicated for TAG-1, which utilizes homophilic binding to promote cell aggregation and heterophilic binding to L1 and β_2 integrin for enhancing neurite outgrowth (Felsinfeld et al., 1994).

The mechanism by which LAMP participates in the formation of limbic circuits is still unclear but, because of its location both pre- and postsynaptically during development, it is possible that it can specifically facilitate interactions with select neurons in the CNS during development. The previous antibody perturbation studies and the present experiments indicate that LAMP is likely to mediate specific recognition through adhesive interactions. Our recent cloning of the gene encoding LAMP will facilitate direct examination of its role during formation of limbic circuits in the mammalian brain.

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GP55 Inhibits both Cell Adhesion and Growth of Neurons, but not Non-neuronal Cells, via a G-protein-coupled Receptor

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Abstract

There is compelling evidence for the role of inhibitory molecules in guiding neurons to their appropriate targets. Furthermore, continued expression of these molecules in the adult could explain why there is little regeneration of neurons in the central nervous system. We have previously identified a family of glycosyl phosphatidylinositol-linked glycoproteins (GP55) from adult chicken brain that has been shown to inhibit neurite outgrowth from dorsal root ganglion and forebrain neurons. GP55 consists of two or more glycoproteins and belongs to a subgroup of the Ig superfamily which contains OBCAM, LAMP, neurotrimin and CEPU-1. We now show that GP55 is anti-adhesive, blocking the adhesion of neurons to normally adhesive substrata in a concentration dependent manner. The anti-adhesive effect can be blocked using antiserum raised against GP55 and pertussis toxin (PTX) but not the β oligomer alone. In contrast, the adhesion of fibroblasts and Schwann cells to the substrata is not affected by GP55. Indeed, non-neuronal cells spread and grow normally. These results would suggest that both the anti-adhesive effect and the inhibition of outgrowth by GP55 is specific to neurons and is mediated by a PTX sensitive, G-protein-coupled receptor.

Introduction

The accurate navigation of neurons to their appropriate target requires that they recognize and respond to navigational cues in their environment. These molecular cues will alter the ability of growth cones to adhere and extend axons. Adhesion to a substrate is necessary for neurite outgrowth and may modulate the rate of neurite elongation. It has been shown that growth cones can respond dramatically to changes in adhesivity of the substrate. The rate of migration increases with increased adhesion. However, on highly adhesive substrata, the migration rate decreases to zero (Burden-Gully *et al.*, 1995). Furthermore, neurite fasciculation *in vitro* and *in vivo* has been described as the result of competition between the growth cone–axon and growth cone–substrata adhesion. (Lemmon *et al.*, 1992). Several molecules have been shown to have an anti-adhesive effect (for review see Chiquet-Ehrismann, 1995). Connectin, a drosophila protein, acts as repulsive signal to motoneurons that innervate the muscle, and has been shown to regulate the extent of innervation (Nose *et al.*, 1994). Another class of molecules that display an anti-adhesive effect are proteoglycans, especially chondroitin sulphate proteoglycans that are present in the brain, spinal cord and along the neural crest pathways during development (Oakley and Tosney, 1991). These include neurocan, phosphocan and versican. Even the normally adhesive molecule laminin has been shown to be anti-adhesive in certain circumstances. Different isoforms of laminin are differentially active in promoting cell adhesion and migration and this is dependent on the neuronal receptor as well as the type of laminin (Rousselle and Aumaillev, 1994). Interestingly, laminin-β2 is thought to provide a specific stop signal. Although laminin-β2 and laminin support motoneuron adhesion and growth cone motility, laminin-β2 is localized in the synaptic region of the muscle fibre basement membrane and provides a specific stop signal that determines the site of synapse formation (Porter *et al.*, 1995). Tenascin C has been shown to affect cell movement in a manner that is different to fibronectin or laminin (Pesheva *et al.*, 1994); The normal spread appearance of fibroblasts is changed and the cells round up. Cellular migration is inhibited and neurons will not invade areas that contain tenascin C. However, tenascin C can also modulate adhesive activity, depending on its expression, and in combination with other extracellular matrix molecules it can either be adhesive or repulsive.

An adhesive interaction between two cells is sufficient to initiate signal transduction pathways that may result in stabilizing the adhesive interaction or the eventual repulsion. In the former case, this can lead to an increase in surface area termed cell spreading, a decrease in the distance between the substratum and the cell and an alteration of cell motility. All these changes within the cell require rearrangement of the cytoskeleton and is mediated by the induction of c-*fos*, c-*myc* and changes in ion fluxes across the membranes (for review see Huttenlocher *et al.*, 1995). There is evidence that non-receptor tyrosine kinases of the *src* family are involved in mediating adhesion and affect outgrowth. It has been shown from *in vitro* experiments, that v-*src* activation increases phosphorylation of cytoskeletal components

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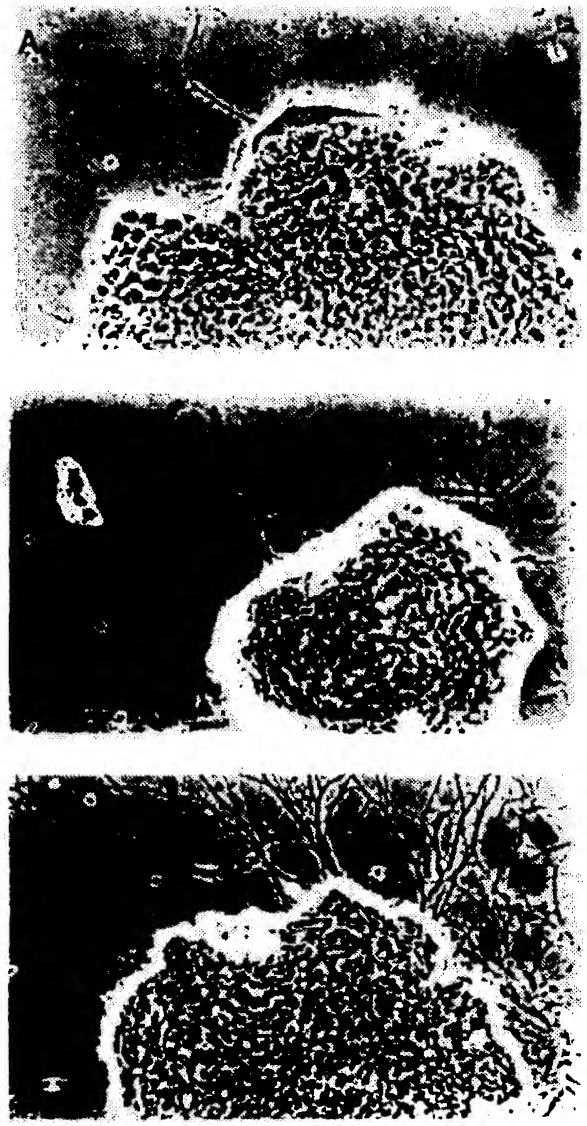


FIG. 1. Soluble GP55 inhibits neurite outgrowth from dorsal root ganglion (DRG) explants. E10 DRG explants were plated out on 10 μg/ml laminin (Ln) and allowed to grow for 6 h. Then 100 μg/ml GP55 was added to selected wells, to others an equivalent volume of Hank's buffered saline solution (HBSS) was added, other wells were fixed after 6 h. The control and experimental explants were incubated for 24 h at 37°C. (A) In the presence of GP55, after 24 h there was little out growth; in fact, there was no difference seen between the explants in the presence of GP55 and those fixed after 6 h. (B) DRG explant fixed after 6 h; in this time the explant has adhered to the substratum and has started to produce neurites. (C) Dorsal root ganglion (DRG) explant growing on laminin after 24 h; note the large number of long neurites emerging from the circumference. Magnification ×160, bar 50 μm.

which correlates with an decrease in adhesion mediated by cadherins and the Ig superfamily (Grumbiner, 1993; Ignelzi *et al.*, 1994).

We have identified a family of glycosyl phosphatidylinositol-linked glycoproteins GP55 which inhibit the extension of neurites and also inhibit the adhesion of neurons to an otherwise favourable substrate. GP55 is isolated from adult chick brain and consists of two (or more) proteins, both of which inhibit the extension of neurites (Clarke and Moss, 1994; Wilson *et al.*, 1996). Two members of the GP55 family have been cloned and sequenced. They belong to a subgroup of the Ig superfamily which is characterized by the possession of three C2 domains and a glycosyl phosphatidylinositol anchor. Other members of the family include: opiate binding cell adhesion molecule (OBCAM) (Schofield *et al.*, 1989), neurotrimin (Struyk *et al.*, 1995), limbic system associated membrane protein (LAMP) (Zhukareva and Levitt, 1995) and a recently discovered chick protein CEPU-1 (Spaltmann and Brummendorf, 1996). The function of most of these molecules is unknown, although LAMP can act as a cell adhesion molecule and will stimulate neurite outgrowth but only from neurons which themselves express LAMP. Our results suggest that members of this subgroup of the Ig superfamily (which has been called the IgLON family) may be able to promote or inhibit cell adhesion and neurite outgrowth depending on the receptors expressed on the neurons. There are a number of precedents for this, including myelin-associated glycoprotein (MAG), which will stimulate outgrowth from embryonic dorsal root ganglion (DRG) neurons but inhibit outgrowth from adult DRG neurons and neurons from the central nervous system (McKerracher *et al.*, 1994). Netrin-1 will also act as a chemoattractant for commissural neurons and a chemorepellant for trochlear motor neurons (Colamarino and Tessier-Lavigne, 1995).

In this paper we present data to show that GP55 is anti-adhesive for neuronal cells but has no effect on fibroblast or Schwann cell adhesion and spreading, indicating that the anti-adhesive effect of GP55 is neuronal specific. This effect can be reversed using antiserum to GP55 and pertussis toxin (PTX) (although not the β oligomer of PTX alone), suggesting that action of GP55 is mediated by a G protein-coupled receptor. These results have implications for the role of GP55 in guidance and the maintenance of axon fascicles and synapses.

Materials and methods

Phosphatidylinositol phospholipase C was purchased from Boehringer Mannheim (Lewes, UK). All tissue culture reagents and tissue culture plastic were provided by Life Technologies (Paisley, UK), nitrocellulose [Schleicher and Schuell (BA85)] was purchased from Anderman (Kingston-Upon-Thames, UK), the β oligomer of PTX was obtained from Calbiochem-Novabiochem (Nottingham, UK), PTX and all other reagents were purchased from Sigma (Poole, UK). The polyclonal antiserum against GP55 was raised in our own laboratory. G4 was a generous gift from Dr E.J de la Rosa (Instituto Cajal CSIC, Madrid, Spain).

Preparation of GP55

GP55 was isolated from adult chicken brain membrane skeleton as described previously (Clarke and Moss, 1994). Briefly, the membrane skeleton was digested with 50 mU/ml phosphatidylinositol phospholipase-C for 2 h at 37°C, in the presence of protease inhibitors. The 100 000 *g* supernatant was applied to a concanavalin A (Con A) affinity column. The GP55 peak was then run on a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel to separate out the proteins. The whole of the GP55 containing band was excised and electroeluted as described before (Wilson *et al.*, 1996). A slice of non-protein-containing, polyacrylamide gel was also electroeluted as a control to eliminate any artefact in the adhesion assays due to the preparation of the protein.

Cell culture

Dorsal root ganglion (DRG) cells were dissected from E9–E11 chick embryos, dissociated as described previously (Clarke and Moss,

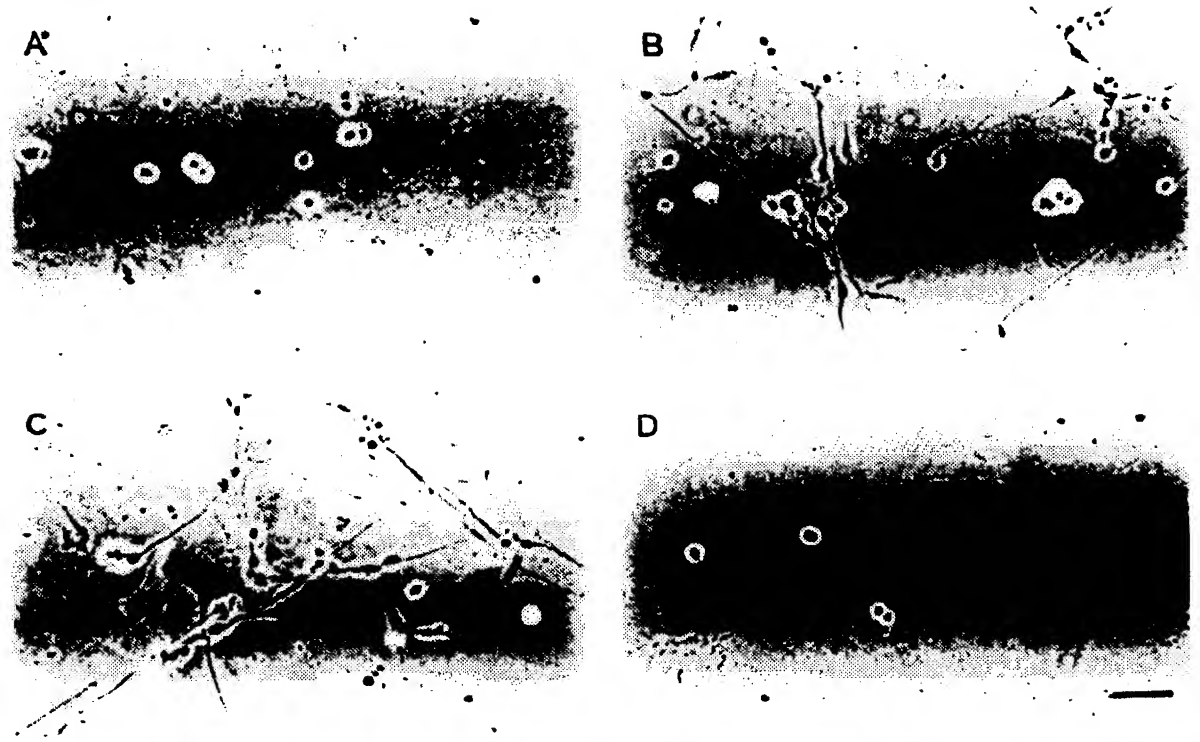


FIG. 2. Pertussis toxin (PTX) but not the β oligomer reverses the inhibitory effect of GP55 on dorsal root ganglion (DRG) outgrowth. (A) DRG neurons plated out on 100 μg/ml laminin plus 10 μg/ml GP55 did not produce any neurites and very few stuck to the substratum. (B) DRG neurons plated out on laminin plus GP55 in the presence of 100 ng/ml PTX grew well and outgrowth was comparable to that seen on laminin alone. (C) DRG neurons plated out on laminin; note the characteristic long neurites and extensive growth. (D) DRG neurons plated out on laminin plus GP55 in the presence of 100 ng/ml β oligomer of PTX; the β oligomer did not reverse the inhibitory effect of GP55 on DRG neurite outgrowth. Magnification ×160, bar 50 μm.

1994), grown in L15 medium plus Glutamax I™, with 10% fetal calf serum, 0.5% methylcellulose, 0.06% glucose, 100 IU penicillin, 100 μg/ml streptomycin and 100 ng/ml nerve growth factor, in air at 37°C. Forebrain neurons were prepared from E7–E9 chick embryos then cultured in Dulbecco's modified Eagle's medium (DMEM):F12 (Ham) medium, 1:1 ratio, plus Glutamax I™ with 0.06% glucose, 0.05 mg/ml transferrin, 0.2 M insulin, 100 IU penicillin, 100 μg/ml streptomycin, at 5% CO_2, 37°C, as described previously (Clarke and Moss, 1994). Cells were grown on 13 mm coverslips that had been coated with 15 μl of nitrocellulose, dissolved in methanol, air dried, coated with 10 μl of 10 μg/ml laminin or poly-L-lysine for 30 min, blocked with 1% bovine serum albumin (BSA) for 30 min, then washed in Hank's balanced salt solution (HBSS). For mixed substrate assays the second protein was applied for 30 min before blocking with BSA. In experiments using the antiserum a 1/50 dilution of the antiserum in 1% BSA/PBS was applied to the substratum before being blocked. Pre-immune rat serum was used as a control at the same dilution. PTX and the β oligomer of PTX (β oligomer) were added to the culture medium to a final concentration of 100 ng/ml.

The chick heart fibroblasts were obtained from E6 chick embryo heart cultured in DMEM with 10% fetal calf serum, 100 IU penicillin and 100 μg/ml streptomycin.

Chick Schwann cells were prepared from a E15 sciatic nerve. The mouse Schwann cells were a gift from Dr D. Edgar (University of Liverpool, UK). All non-neuronal cells were maintained in DMEM with 10% fetal calf serum, 100 IU penicillin and 100 μg/ml streptomycin, at 10% CO_2 and 37°C.

The effect of soluble GP55

Explants were plated out on 10 μg/ml laminin substrata. After 6 h GP55 was added to the culture medium to a final concentration of 100 μg/ml. The explants were fixed in Luduena's fixative after 6 h and 24 h. These were compared to explants grown without GP55 and fixed at the same time points.

Cell adhesion assay

The adhesion assay was performed as described previously (Clarke and Moss, 1994). Briefly, dissociated DRG or forebrain neurons were plated out on various substrata as described above. After a period of 2 h, the unbound cells were removed by gently washing each coverslip with three changes of PBS, before being fixed with Luduena's fixative. The coverslips were mounted and the remaining phase bright spherical cell bodies were observed under phase contrast microscopy and counted in three fields (1 mm^2); the mean number of cell bodies was determined for each substratum. Statistical analysis was performed using the analysis of variance test.

Results

The inhibitory effect of GP55 on DRG outgrowth has been demonstrated previously (Clarke and Moss, 1994). Experiments designed to

measure the inhibition of neurite outgrowth that depend entirely on substrate bound assays, have been open to some criticism. We have therefore tested the ability of soluble GP55 to arrest the growth of established neurites from DRG explants. The explants were plated out on laminin 6 h before the application of 100 μg/ml GP55. In the presence of GP55 (Fig. 1A) after 24 h there was little outgrowth. The outgrowth in the presence of GP55 after 24 h was similar to that observed with the control explant growing on laminin that had been fixed after 6 h (Fig. 1B). In contrast, the control explant after 24 h displayed extensive outgrowth with numerous neurites emerging from the circumference of the explant (Fig. 1C). We have reported previously that PTX will block the activity of GP55. However, the mechanism by which PTX modulates growth cone guidance has been questioned (Kindt and Lander, 1995). In order to discover whether the inhibition of DRG outgrowth by GP55 is reversed by the

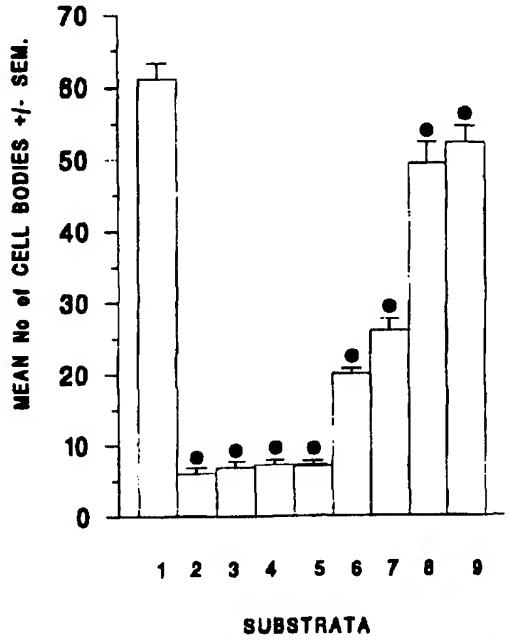
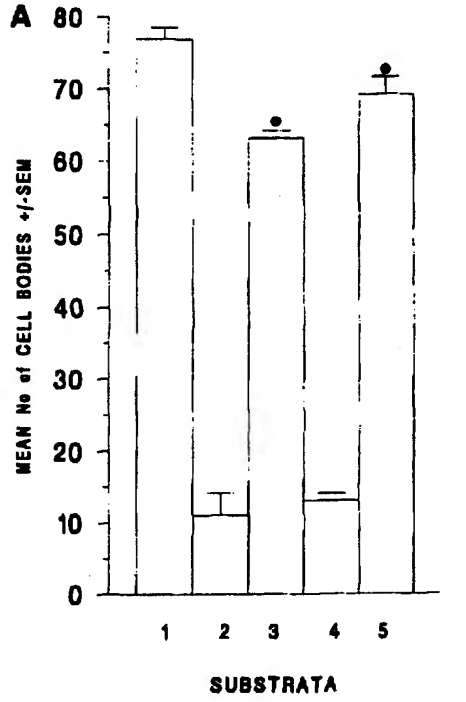


FIG. 3. Demonstration of the anti-adhesive effect of GP55 on dorsal root ganglion (DRG) cell adhesion. The attachment of cell bodies to coverslips coated with 10 μg/ml laminin in the presence of various concentrations of GP55 was determined after 2 h. (1) control, laminin (Ln); (2) Ln + 25 μg/ml GP55; (3) Ln + 10 μg/ml GP55; (4) Ln + 5 μg/ml GP55; (5) Ln + 1 μg/ml GP55; (6) Ln + 0.5 μg/ml GP55; (7) Ln + 0.1 μg/ml GP55; (8) Ln + 0.05 μg/ml GP55; (9) Ln + 0.01 μg/ml GP55. The number of bound cells was calculated (1 mm^2 field) in triplicate for each coverslip. ● indicates significance ($P < 0.001$, $n = 9$). These data show that GP55 inhibits DRG cell adhesion to laminin in a concentration dependent manner.



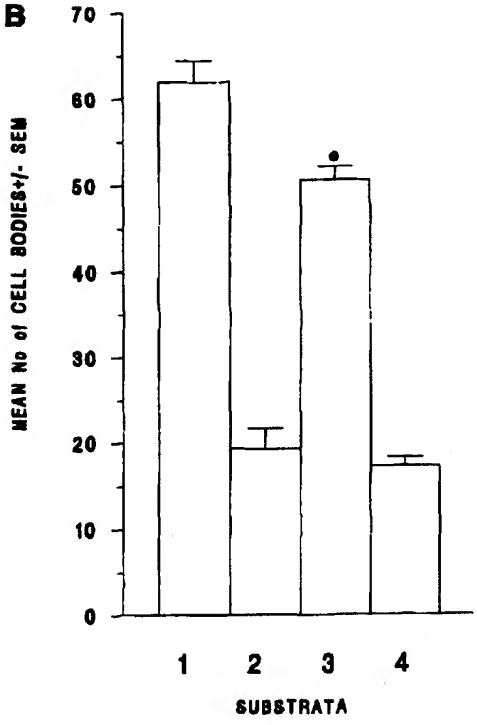


FIG. 4 (A) Antiserum raised against GP55 reverses the anti-adhesive effect of GP55 on dorsal root ganglion (DRG) cell adhesion. (1) Control, 10 μg/ml laminin (Ln) + 1/50 pre-immune rat serum; (2) Ln + 1 μg/ml GP55 + 1/50 pre-immune rat serum; (3) Ln + 1 μg/ml GP55 + 1/50 GP55 antiserum; (4) Ln + 0.5 μg/ml GP55 + 1/50 pre-immune rat serum; (5) Ln + 0.5 μg/ml GP55 + 1/50 GP55 antiserum. The number of cell bodies per field (1 mm^2) after 2 h was determined for each coverslip in triplicate; ● indicates significance ($P < 0.001$, $n = 9$). The antiserum raised against GP55 was able to significantly reverse the anti-adhesive effect of GP55, restoring the number of cell bodies to control levels. (B) The anti-adhesive effect of GP55 can be blocked by pertussis toxin (PTX). (1) Control, 10 μg/ml laminin (Ln); (2) Ln + 0.5 μg/ml GP55; (3) Ln + 0.5 μg/ml GP55 + 100 ng/ml PTX; (4) Ln + 0.5 μg/ml GP55 + 100 ng/ml β oligomer. The number of cell bodies, after 2 h, was calculated per field (1 mm^2), in triplicate for each coverslip; ● indicates a significant difference ($P < 0.001$, $n = 9$). PTX blocks the anti-adhesive effect of GP55 on DRG cells, but there was no significant increase in adhesion in the presence of the β oligomer of PTX.

conventional action of PTX-G protein ribosylation, we used the β oligomer of PTX (β oligomer) as a negative control. The β oligomer does not contain the A protomer that has the ADP ribosylating activity. In the presence of GP55 there was no neurite outgrowth (Fig. 2A). This was reversed in the presence of 100 ng/ml PTX and outgrowth (Fig. 2B) was comparable to that on laminin (Fig. 2C). The β oligomer (100 ng/ml) did not reverse the effect of GP55 (Fig. 2D); there was no outgrowth observed. Quantitation of this experiment revealed that PTX increased the neurite outgrowth, on laminin/GP55, from 0% to 49.1 ± 1.4% (in contrast to 78.6 ± 1.9% on laminin alone), while the β oligomer had no effect. Even at concentrations up to 500 μg/ml β oligomer gave no increase in neurite outgrowth (data not shown).

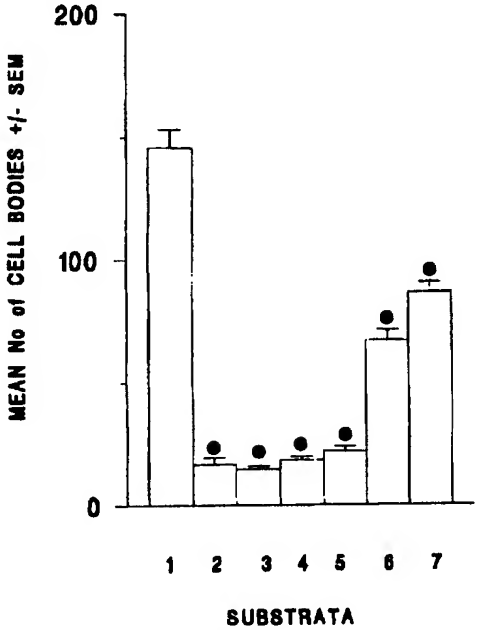


FIG. 5. Demonstration of the anti-adhesive effect of GP55 on forebrain cell adhesion. The attachment of cell bodies to coverslips coated with 10 μg/ml poly-L-lysine (PL) and various concentrations of GP55 was determined after 2 h. (1) Control, PL; (2) PL+ 25 μg/ml GP55; (3) PL+ 10 μg/ml GP55; (4) PL + 5 μg/ml GP55; (5) PL + 1 μg/ml GP55; (6) PL + 0.5 μg/ml GP55; (7) PL + 0.1 μg/ml GP55. The number of bound cells was calculated per field (1 mm^2) in triplicate for each coverslip. ● indicates a significant difference ($P < 0.001$, $n = 9$). The inhibition of forebrain cell adhesion by GP55 is concentration dependent.

We have shown in a preliminary study (Clarke and Moss, 1994) that GP55 has an anti-adhesive effect on DRG and forebrain neurons. We have investigated this observation further. We examined the concentration dependence of GP55 on DRG cell adhesion to a laminin substratum and found that GP55 had a more potent effect on cell

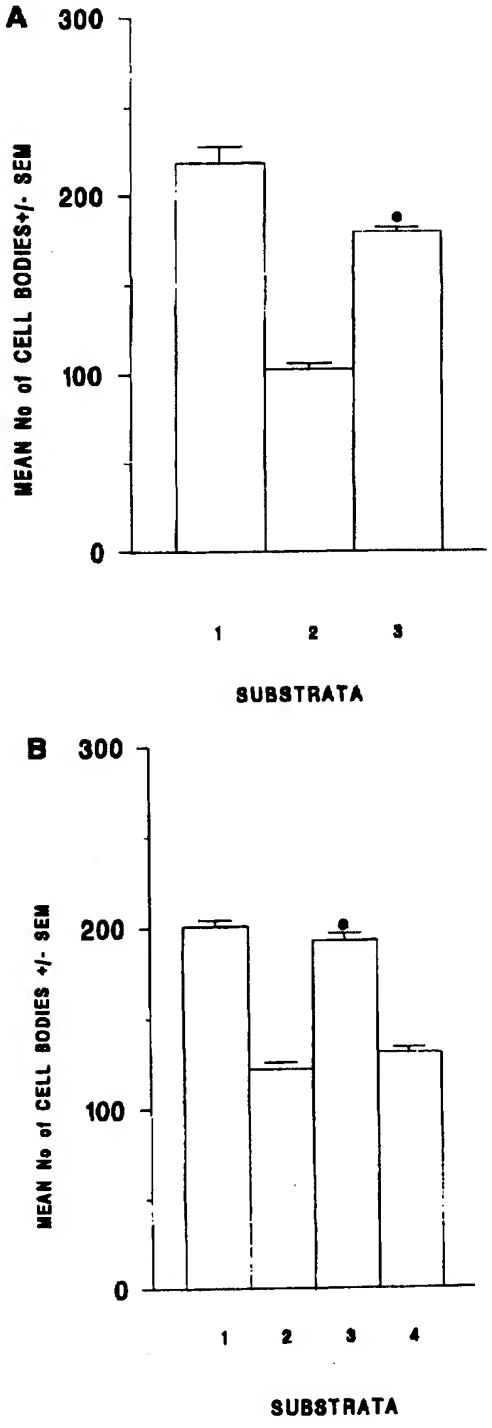


FIG. 6 (A) Antiserum to GP55 restores forebrain cell adhesion. (1) Control, 10 μg/ml poly-L-lysine (PL) + 1/50 pre-immune rat serum; (2) PL+ 0.5 μg/ml GP55 + 1/50 pre-immune rat serum; (3) PL + 0.5 μg/ml GP55 + 1/50 GP55 antiserum. The number of cell bodies per field (1 mm^2) after 2 h, was calculated in triplicate for each coverslip. ● indicates significant difference ($P < 0.001$, $n = 9$). The antiserum significantly increased adhesion in the presence of GP55. (B) Pertussis toxin (PTX) reverses the anti-adhesive effect of GP55 on forebrain neurons. (1) control, 10 μg/ml poly-L-lysine (PL); (2) PL+ 0.5 μg/ml GP55; (3) PL + 0.5 μg/ml GP55 + 100 ng/ml PTX; (4) PL + 0.5 μg/ml GP55 + 100 ng/ml β oligomer of PTX. The number of cells bound after 2 h was calculated per field (1 mm^2) in triplicate for each coverslip. ● indicates a significant difference ($P < 0.001$, $n = 9$). PTX blocks the anti-adhesive effect of GP55 on forebrain neurons, but there was no significant increase in adhesion in the presence of the β oligomer.

adhesion than on the inhibition of outgrowth. The EC_{50} for anti-adhesion was between 50 and 100 ng/ml GP55 (Fig. 3); in contrast, the EC_{50} for inhibition of outgrowth was between 5 and 10 μg/ml GP55 (Clarke and Moss, 1994). The antiserum raised against GP55 was able to block the effect of GP55 on cell adhesion (Fig. 4A). In the presence of the antibody there was a significant increase in the number of cell bodies that bind to the substrata. We also tested the possibility that the antiserum could enhance adhesion of DRG neurons to the substrata directly. We found that there was no significant enhancement of adhesion compared to pre-immune serum ($P > 0.05$, $n = 9$, data not shown).

Because a PTX-sensitive G protein may mediate the inhibition of neurite outgrowth in response to GP55, we then assessed the effect of PTX on the anti-adhesive properties of GP55. In the presence of 100 ng/ml PTX there was a significant increase in the number of cell bodies on a laminin/GP55 substrate (Fig. 4B), typically restoring the number of cell bodies to 82% of the control. The negative control, β oligomer, had no significant effect on the number of cell bodies (Fig. 4B), suggesting that the restoration of adhesion is due to a specific effect of PTX and not the membrane binding properties of the β oligomer.

The effect of GP55 on the adhesion of forebrain neurons to various substrata was also assessed. It was found that GP55 had an anti-adhesive effect, that was concentration dependent (Fig. 5). The EC_{50} was about 100 ng/ml GP55 (Fig. 5). The anti-adhesive effect of GP55 on forebrain neurons is again blocked by the antiserum (Fig. 6A). Indeed, there is a significant increase in the number of cell bodies on poly-L-lysine/GP55 substratum in the presence of the antibody, yielding a cell count that was 82% of the control. There was no significant enhancement of forebrain cell adhesion on the antibody substratum compared to the control pre-immune serum ($P > 0.05$, $n = 9$, data not shown). As seen with DRG neurons, the anti-adhesive effect of GP55 on forebrain neurons can be reversed using PTX (Fig. 6B), once again the β oligomer gave no significant increase in the number of cell bodies attached to the substratum (Fig. 6B).

As a further control, DRG were also plated out on the naturally occurring chick cell adhesion molecule, G4, to show that GP55 was not specifically interfering with binding to laminin substrata. GP55 inhibited neuronal adhesion to G4 in a similar manner to that observed using laminin as the substrate (Fig. 7). The anti-adhesive effect of GP55 on cell adhesion on G4 substratum, was concentration dependent with an EC_{50} of between 50 and 100 ng/ml GP55.

At the end of some cell adhesion experiments the cells that did not adhere were removed and re-plated on laminin substrata with an island of 25 μg/ml GP55 to assess the effect of their outgrowth after 18 h. The re-plated cells grew well on laminin but did not grow well on GP55. Hence, the effect of GP55 is unlikely to be cytotoxic (unpublished observation).

So far, all the data obtained for GP55 has been concerned with its effect on neuronal cells; we wanted to assess the effect of GP55 on non-neuronal cells. We plated out mouse Schwann cells and chick heart fibroblasts on various substrata. We found that GP55 did not have an anti-adhesive effect on cell adhesion (Fig. 8); there was no significant difference between the control cells plated on poly-L-lysine and poly-L-lysine/GP55 substratum. We also found that the non-neuronal cells started to spread on poly-L-lysine/GP55 substrata after 2 h. Indeed, chick Schwann cells and chick heart fibroblasts grown overnight on 100 μg/ml GP55 spread and eventually become confluent (Fig. 9); at 100 μg/ml GP55, neuronal cells will not adhere and there is no observed outgrowth (Clarke and Moss, 1994). These data suggest that the anti-adhesive effect of GP55 on cell adhesion may be specific to neurons and not due to a charge anomaly of the substrate.

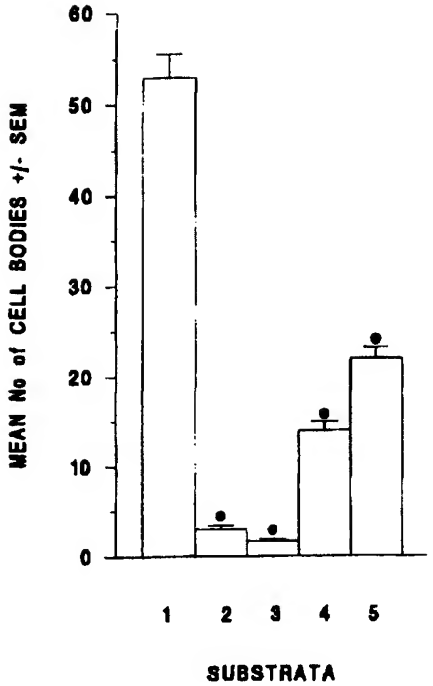


FIG. 7. The anti-adhesive effect of GP55 on DRG cell adhesion is not substrate dependent. The effect on dorsal root ganglion (DRG) adhesion to G4 substrata in the presence of various concentrations of GP55 was determined after 2 h. (1) Control, 10 μg/ml G4; (2) G4 + 25 μg/ml GP55; (3) G4 + 5 μg/ml GP55; (4) G4 + 1 μg/ml GP55; (5) G4 + 0.5 μg/ml GP55. The number of cells bound was determined per field (1 mm^2) in triplicate for each coverslip. ● indicates a significant difference ($P < 0.001$, $n = 9$). The anti-adhesive effect of GP55 on DRG adhesion to G4 substrata was shown to be concentration dependent.

Discussion

We have shown in this paper that GP55 will act as an anti-adhesive molecule. The results we observe are probably due to a specific receptor expressed on neurons rather than any non-specific effect. Since it has a pI between 6 and 7 (D. Wilson and D. Moss, unpublished results), GP55 does not have an unusual negative charge and this argues against a non-specific charge effect. In addition, not all cells respond to GP55. Chick heart fibroblasts, chick Schwann cells and mouse Schwann cells appear able to grow and spread on GP55 alone, even at a concentration that completely blocks both adhesion and growth of neurons. This is in contrast to NI-35 which does stop fibroblast spreading (Caroni and Schwab, 1988). GP55 will also block adhesion of neurons to a variety of substrata including G4, laminin and poly-L-lysine. Thus, GP55 is unlikely to be sterically blocking the interaction between substrate molecules and their receptor.

Further evidence for a specific receptor comes from the results using PTX. We have shown previously that GP55 blocks neuronal cell adhesion and neurite outgrowth from DRG and forebrain neurons and that this inhibition is reversed by PTX (Clarke and Moss, 1994). It has since been reported that PTX may be able to inhibit growth cone guidance mechanisms by a G protein independent mechanism

which depends only on the β oligomer subunit of PTX (Kindt and Lander, 1995). We show here that PTX will almost completely block the activity of GP55 at 100 ng/ml and that the β oligomer alone at up to 500 ng/ml has no effect. Hence, the β oligomer alone will reverse neither the inhibition of neurite outgrowth nor the anti-adhesive activity of GP55. The same results are observed for both DRG and forebrain neurons. These results are compatible with PTX acting in its normal role, as an inhibitor of G proteins by ADP ribosylation. This suggests that both the inhibition of neurite outgrowth and the anti-adhesive effect of GP55 may be mediated via a G protein-coupled receptor. The differing sensitivity of neuronal response to GP55 of these two closely related phenomena is surprising and requires further investigation. However, in our earlier experiments we were measuring neurite outgrowth from a population of neurons which did adhere to GP55 and were perhaps less sensitive to GP55. This would not be entirely surprising, since both forebrain neurons and DRG neurons consist of many different cell types.

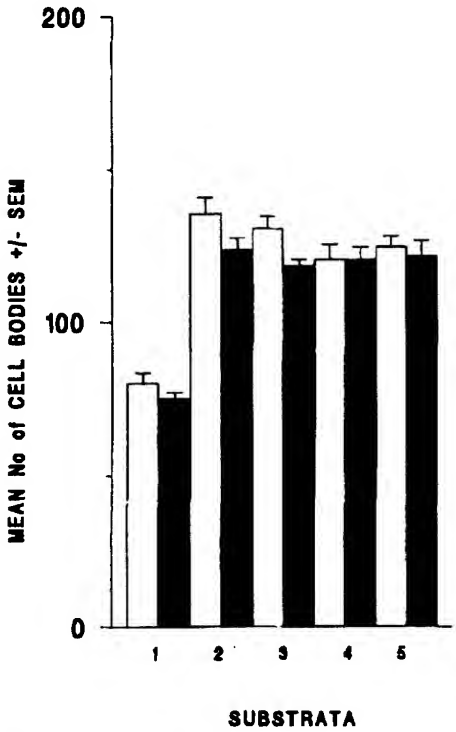


FIG. 8. The effect of GP55 on non-neuronal cell adhesion. Mouse Schwann cells and chick heart fibroblasts were plated out on either 100 μg/ml GP55 or 10 μg/ml poly-L-lysine with various concentrations of GP55. The number of cells bound after 2 h was then determined. Open bars, mouse Schwann cells; closed bars, chick heart fibroblasts plated out on: (1) GP55; (2) PL; (3) PL + 25 μg/ml GP55; (4) PL + 10 μg/ml GP55; (5) PL + 1 μg/ml GP55. The number of cells bound per field (1 mm^2) was calculated in triplicate for each coverslip. There was no apparent difference between cell adhesion on poly-L-lysine and poly-L-lysine/Gp55 substrata. GP55 does not appear to have an anti-adhesive effect on non-neuronal cells.

The possibility that cell adhesion is mediated by signal transduction events is well established (for reviews see Huttenlocher *et al.*, 1995; Muller and Kypta, 1995). Thus, tenascin R displays repellent substrate properties towards neurons and this effect is time dependent (Lochter *et al.*, 1991). When first plated out cerebellar neurons adhere to tenascin R substratum, this initial adhesion is followed by a gradual loss of cell–substratum adherence. The remaining cells tend to aggregate and the neurons fasciculate. This effect has been shown to be mediated by F3/11 and it is the interaction with F3/11 that is responsible for the initial binding (Pesheva *et al.*, 1993). In our case events are more rapid, and even in 2 h signal transduction mechanisms must occur which allow neurons to stabilize their interaction with the substrate. It is possible that GP55 may be interfering with the normal signal transduction pathways that are required to stabilize adhesive interactions in neuronal cells.

The experimental evidence to implicate the G proteins in the transduction of inhibitory cues and in growth cone collapse is unclear. However, there is evidence that Go is involved in mediating growth cone collapse, the G protein Go has been shown to be the most prominent G protein in the growth cone (Strittmatter *et al.*, 1990), and peptides from the N terminus of GAP-43 will bind to Go. When introduced into chick DRG neurons these will cause growth cone collapse (Strittmatter *et al.*, 1994; Igarashi *et al.*, 1995). It remains to be seen whether guidance cues such as NI-35 and collapsin also act via G proteins.

GP55 consists of two (or more) related proteins, both of which have been shown to inhibit neurite outgrowth (Wilson *et al.*, 1996). Two clones corresponding to GP55 have been sequenced. GP55 is related to OBCAM, neurotrimin, LAMP and a recently discovered chick molecule, CEPU-1. These glycoproteins are thought to act as

FIG. 9. Non-neuronal cells spread and grow on GP55 at concentrations that inhibit neuronal cell adhesion and outgrowth. Non-neuronal cells were plated out on 100 μg/ml GP55 and left for 48 h; the cells were then fixed in Ludueña's fixative and photographed under phase contrast microscopy. (a) Chick heart fibroblasts; (b) chick Schwann cells. Both fibroblasts and Schwann cells display a spread appearance and grow normally on GP55. Magnification ×160, bar 50 μm.

markers for specific classes of neurons and most have a restricted distribution within the nervous system. Our results demonstrate the ability of some members of this family to inhibit the growth and adhesion of neurons. This raises the attractive hypothesis that this family of proteins may play a role in determining the specificity of neuron–neuron interactions. Thus, members of the GP55 family may permit the interaction of the correct neurons whilst repelling the interaction of incorrect neurons. In keeping with this hypothesis, LAMP will encourage growth and fasciculation of neurons which also express LAMP (Zhukareva and Levitt, 1995), whilst we have shown that GP55 will inhibit the growth and adhesion of neurons which do not express GP55 (Clarke and Moss, 1994).

Acknowledgements

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Abbreviations

BSA	bovine serum albumin
β oligomer	β oligomer of pertussis toxin-negative control
DRG	dorsal root ganglion
DMEM	Dulbecco's modified Eagle's medium
LAMP	limbic system associated membrane protein
Ln	laminin
OBCAM	oplate binding cell adhesion molecule
PTX	pertussis toxin

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The Limbic System–Associated Membrane Protein Is an Ig Superfamily Member That Mediates Selective Neuronal Growth and Axon Targeting

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Summary

The formation of brain circuits requires molecular recognition between functionally related neurons. We report the cloning of a molecule that participates in these interactions. The limbic system–associated membrane protein (LAMP) is an immunoglobulin (Ig) superfamily member with 3 Ig domains and a glycosyl-phosphatidylinositol anchor. In the developing forebrain, *lamp* is expressed mostly by neurons comprising limbic-associated cortical and subcortical regions that function in cognition, emotion, memory, and learning. The unique distribution of LAMP reflects its functional specificity. LAMP-transfected cells selectively facilitate neurite outgrowth of primary limbic neurons. Most striking, administration of anti-LAMP in vivo results in abnormal growth of the mossy fiber projection from developing granule neurons in the dentate gyrus of the hippocampal formation, suggesting that LAMP is essential for proper targeting of this pathway. Rather than being a general guidance cue, LAMP likely serves as a recognition molecule for the formation of limbic connections.

Introduction

Activity independent and dependent mechanisms underlie the generation of precise neuronal connections (Goodman and Shatz, 1993). The former is probably mediated by common recognition strategies utilized by all organisms (Hynes and Lander, 1992; Jessell and Melton, 1992; Goodman, 1994), with guidance cues provided by adhesion molecules operating in combination with diffusible chemotropic factors released from restricted populations of guiding or target cells and molecules providing repulsive cues (Tessier-Lavigne, 1994). Ubiquitously expressed members of the immunoglobulin superfamily (IgSF), integrins and cadherins, can modulate growth of all types of neurons (Jessell, 1988), but it is thought that those molecules expressed uniquely among groups of functionally related neurons are more likely to provide specific information regarding selective growth cone guidance leading to correct targeting.

Biochemical and immunocytochemical mapping studies identified a 64–68 kDa glycoprotein, the limbic system–associated membrane protein (LAMP), that is expressed by cortical and subcortical neurons comprising the limbic system (Levitt, 1984; Zacco et al., 1990). These brain areas form functional circuits involved in memory, learning, cognitive behavior, and central autonomic regulation. LAMP immunoreactivity is present early during development on neurons and transiently on growth cones and axons during pathway formation and differentiation (Horton and Levitt, 1988; Keller and Levitt, 1989; Zacco et al., 1990). From the unique patterns of LAMP immunoreactivity, we suggested that LAMP may serve as a critical component of recognition during circuit formation in the mammalian brain.

Considering the potentially important role that LAMP may play in vertebrate brain development, we undertook the present study to characterize its molecular and functional properties. We report the isolation of cDNA clones with the complete coding region of LAMP and characterize the protein as a new member of the IgSF with a high degree of homology to two other proteins containing a glycosyl-phosphatidylinositol (GPI) anchor and three-domain Ig structure. LAMP exhibits some features typical of cell adhesion molecules (CAMs), but is thus far unique among vertebrate CAMs in its ability to selectively induce growth of limbic neurons. This specificity of function is likely to be required for the normal development of an intrahippocampal pathway, as is evident by in vitro studies demonstrating selective adhesive properties and in vivo perturbation experiments administering LAMP antibodies postnatally.

Results

LAMP Is a New Member of the IgSF

Two separate preparations of affinity-purified LAMP yielded N-terminal sequences of 18 and 21 amino acids (Figure 1C, underlined), which were used to design degenerate primers. Four cDNA clones encoding LAMP were isolated by screening a cDNA library from adult rat hippocampus. These were subsequently amplified, subcloned, and sequenced, showing identical inserts of 1238 bp. The sequence of the *lamp* cDNA contains an open reading frame of 1014 bp that starts at the first methionine codon within a strong consensus sequence for initiation of translation (Kozak, 1987).

The predicted protein sequence derived from the open reading frame produces a 338 amino acid polypeptide (Figures 1A and 1C) with a calculated molecular mass of

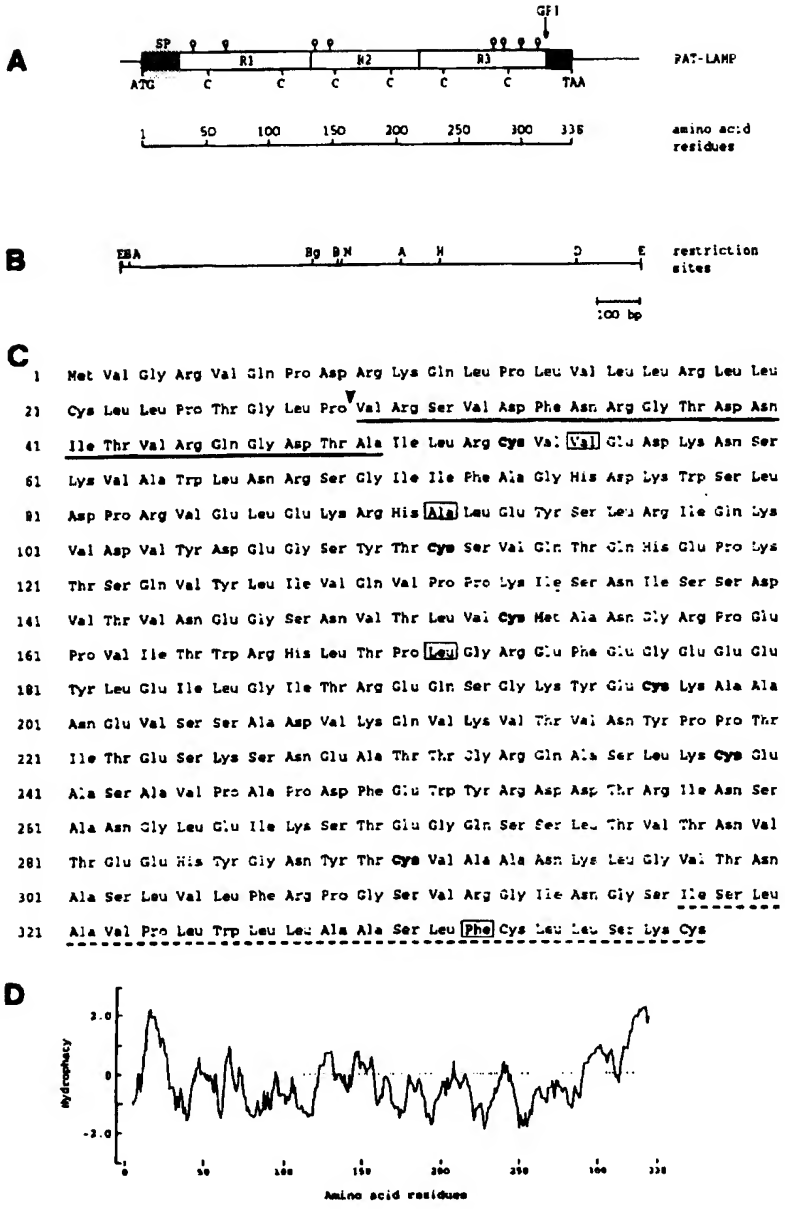

Figure 1. The Nucleotide Sequence of *lamp* cDNA Encodes a Polypeptide with Three Ig-like Domains and a GPI Anchor

(A) Schematic representation of *lamp* mRNA. The mRNA is represented in the 5′ to 3′ orientation with its protein coding region (1014 bp) shown as an open box. The translated open reading frame predicts a 338 amino acid polypeptide with a signal peptide (SP, hatched box) and a hydrophobic C-terminus (closed box) required for the processing of GPI-anchored molecules. LAMP contains three internal repeats (R1–R3), similar to the Ig domains, with conserved pairs of cysteine residues (C). There are eight putative N-glycosylation sites (open circles).

(B) Selected restriction sites are as follows: A, AccII; B, BalI; Bg, BglI; D, DraI; E, EcoRI; H, HindIII; N, NarI.

(C) Predicted amino acid sequence of rat *lamp* cDNA. The N-terminal amino acid sequence of the native protein, obtained by microsequencing, is underlined (thick line) with the predicted cleavage site for the signal peptide denoted by the arrowhead. The conserved cysteine residues of the Ig domains are shown in bold, and the hydrophobic C-terminal sequence is indicated by a dashed line. The amino acid substitution in the human sequence is indicated by the open box.

(D) Hydrophobicity analysis of LAMP predicted amino acid sequence indicates a hydrophilic polypeptide with hydrophobic domains representing the N-terminal signal peptide and the C-terminal sequence.

37 kDa. Hydrophobicity analysis (Kyte and Doolittle, 1982) indicates that the protein is mostly hydrophilic, with hydrophobic domains at both the amino and carboxyl ends (Figure 1D). The hydrophobic N-terminal sequence (amino acids 2–28) has the characteristics of a signal peptide with a predicted cleavage site after Pro28 (Figure 1C, arrowhead; von Heijne, 1986). Consistent with proteolytic processing of the signal peptide, Val29 would become the N-terminal amino acid of the mature protein followed by a predicted amino acid sequence identical with that determined for the purified native protein by microsequencing (Figure 1C). The C-terminus of LAMP contains a short stretch of hydrophobic amino acids (Figure 1C, dashed line), which is a common feature of proteins that are linked to the plasma membrane by a GPI anchor. Generally, this hydrophobic domain is removed by cleavage at a site of a small amino acid, which for LAMP is probably Asn315 (Ferguson and Williams, 1988; Cross, 1990; Gerber et al., 1992), with concomitant addition of the GPI moiety to the C-terminus. Further evidence that LAMP is GPI anchored is provided by the release of the recombinant protein expressed on the surface of transfected CHO cells by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment (see Figure 4B). This agrees with previous biochemical evidence for linkage of native LAMP to the neuronal membrane via a GPI anchor (Zhukareva and Levitt, 1995).

The predicted molecular mass of LAMP after cleavage of the signal peptide and attachment of the GPI anchor

Table 1. Homology of LAMP with Immunoglobulin Superfamily Molecules

	Residues of Protein	Residues of LAMP	Percentage Identity	Opt. Score	z value
OBCAM	5–338	3–332	55.2	983	94.7
Neurotrimin	3–343	1–337	54.6	936	101.1
Amalgam	77–324	71–307	31.3	332	9.3
MAG	234–407	126–305	30.4	221	9.5
Lachesin	14–319	17–306	29.1	342	11.0
N-CAM	260–499	86–310	27.0	282	20.0
SMP	229–404	122–303	25.7	193	12.3
Tag-1	274–505	77–305	25.5	234	9.4
Fas II	1–317	2–305	23.9	247	9.3
L1	247–500	37–294	21.3	180	11.6
Ng-CAM	405–670	16–289	21.3	140	11.9

A search of the Swissprot database with the amino acid sequence of LAMP using the program FASTA (Williams, 1987) revealed significant similarity to cell adhesion molecules of the IgSF. The percentage of amino acid identity between LAMP and these proteins in their most homologous regions are shown. The greatest degree of similarity was found between LAMP and bovine and rat OBCAM and neurotrimin amino acid sequences. The significance of the homology is indicated by the Z value; Z values >6 are probably significant, and Z >10 are definitively significant.

is 32 kDa, considerably lower than the apparent molecular mass of 64–68 kDa determined for the native glycoprotein by SDS-PAGE (Zacco et al., 1990). The recombinant protein produced in E. coli has an apparent molecular mass similar to the predicted core protein (data not shown). The presence of eight putative N-linked glycosylation sites (Asn-X-Ser/Thr) (Figure 1A) suggests that extensive glycosylation of the protein backbone may account for the size discrepancy. Indeed, the recombinant protein, when produced in eukaryotic cells (CHO), has an apparent molecular mass similar to that of the native protein purified from the hippocampus (see Figure 4B). In erythropoietin, for example, the presence of oligosaccharides account for 50% of the protein molecular weight (Takeuchi and Kobata, 1991). The LAMP core protein also contains 11 potential phosphorylation sites (data not shown).

The deduced amino acid sequence contains three internal repeats of 99, 85, and 92 amino acids, 18%–26% homologous to each other in pairwise comparisons, with the highest identity centered around pairs of conserved cysteine residues that resemble those found in the Ig domains of the immunoglobulins and members of the IgSF (Williams, 1987; Williams and Barclay, 1988). Amino acid sequence comparisons using the FASTA program (Pearson and Lipman, 1988) revealed the most significant homologies to be with IgSF CAMs (Table 1). Moderate sequence identity (21%–31%), common among members of the IgSF, was found between LAMP and Amalgam (Seeger et al., 1988), myelin-associated glycoprotein (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987), Lachesin (Karlstron et al., 1993), neural CAM (Cunningham et al., 1987), Schwann cell myelin protein (Dulac et al., 1992), TAG-1 (Furley et al., 1990), Fasciclin II (Grenningloh et al., 1991), L1 (Moos et al., 1988), and Ng-CAM (Burgoon et al., 1991). In contrast, we identified unusually high sequence identity (approximately 55%) to two IgSF members, which also have three Ig-like domains and a GPI anchor, the bovine and rat opioid-binding CAM (OBCAM; Lippman et al., 1992; Schofield et al., 1989), and neurotrimin (Struyk et al., 1995). Sequence analysis of the three Ig domains of LAMP in relation to other representative members of the IgSF allowed the assignment of these domains to the C2 set, even though the first domain showed some similarity with the variable region consensus (Williams, 1987; Williams and Barclay, 1988). Detailed analysis of the homology of LAMP with OBCAM and neurotrimin showed that domains one and two share 60%–62% identity, and the third Ig domain and the N-terminus share 47%–49% identity. Based on the high homology, we propose that these three molecules represent a new subclass in the IgSF that we designate as IgLONs (LAMP, OBCAM, neurotrimin subfamily).

Conservation of individual IgSF molecules among mammalian species is common. This characteristic, in addition to the distribution of LAMP immunoreactivity in highly conserved limbic regions of the brain, suggests that the LAMP molecule should retain critical structural features in the human. The human homolog of LAMP was cloned by RT-PCR using human cerebral cortex RNA in combination with oligonucleotide primers derived from the rat *lamp* sequence to produce several overlapping PCR products that were subcloned and sequenced. Sequence analysis reveals greater than 99% identity, with only four amino acid substitutions (Figure 1C, boxed residues), one of them located in the cleaved hydrophobic C-terminus. The analysis underscores the high conservation between human and rodent LAMP. Finally, genomic Southern blot analysis shows that in human and rat, a single copy gene encodes LAMP (data not shown).

lamp Exhibits Unique Expression among Limbic-Associated Structures

Northern blot analysis, using an antisense riboprobe, revealed two major transcripts of 1.6 kb and 8.0 kb in adult rat hippocampus, perirhinal cortex, and cerebellum, but none in nonneural tissues (Figures 2A and 2B, lane 1). These two bands were also detected with a 53-mer oligonucleotide probe complementary to a region that has relatively lower homology with other CAMs compared with the riboprobe (Figure 2B, lane 2). The deduced amino acid sequence of LAMP indicates that both the 1.6 and 8.0 kb transcripts could readily encode this protein, and that the

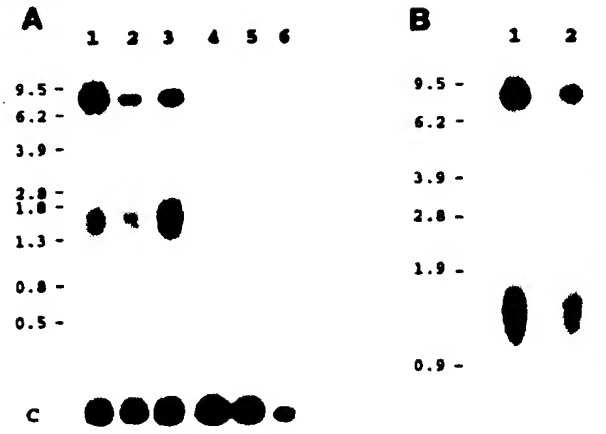

Figure 2. Northern Blot Analysis of *lamp* Gene Expression

(A) Distribution of *lamp* transcripts in adult rat indicates the presence of two transcripts of 1.6 and 8.0 kb in neural tissue (hippocampus [lane 1], perirhinal cortex [lane 2], and cerebellum [lane 3]), but not in nonneural peripheral tissue (kidney [lane 4], lung [lane 5], and liver [lane 6]).

(B) High resolution blots of hippocampal mRNA were hybridized with the antisense riboprobe (lane 1) and with an oligonucleotide (lane 2) derived from a region with minimal homology to OBCAM. Both probes recognized the 1.6 and 8.0 kb transcripts. Cyclophilin probe (c) was used as control for mRNA integrity and amounts of mRNA loaded.

difference between these transcripts resides in the untranslated region. The same bands were also detected with human brain mRNA blots (data not shown).

In situ hybridization using the oligonucleotide probe showed very close, though not identical, correspondence between distribution of the *lamp* transcript and protein immunoreactivity. The general patterns of hybridization indicate relatively high expression in classic limbic regions and, with some exceptions, lower or no detectable expression in primary sensory and motor regions. Structures receiving mixed inputs, such as the striatum, exhibit patterns of hybridization that reflect LAMP immunostaining and known patterns of limbic input (Chesselet et al., 1991). During development, *lamp* hybridization is seen as early as E15–16 in the rat forebrain (Figure 3A), with dense expression in presumptive limbic cortex, basal forebrain, and hypothalamus. There is little hybridization in more dorsal, nonlimbic cortex. Later in gestation, the expression in presumptive limbic cortex remains very high, and medial limbic areas of the thalamus and hypothalamus are also strongly hybridized (Figure 3B). In the adult, the specific distribution of *lamp* transcripts in the cerebral cortex and subcortical limbic areas is similar to patterns seen prenatally (Figure 3C), with very dense expression in perirhinal, hippocampal, cingulate, amygdala, and limbic thalamic neurons and little to no expression in primary sensory areas of the neocortex. In contrast to the immunostaining (Levitt, 1984; Levitt et al., 1986), we found more expression of the *lamp* transcript in sensory thalamic nuclei, including the medial and lateral geniculate, although close examination reveals relatively fewer positive neurons (and less densely hybridized) in these regions than the heavily labeled medial limbic regions or neighboring hypothalamus.

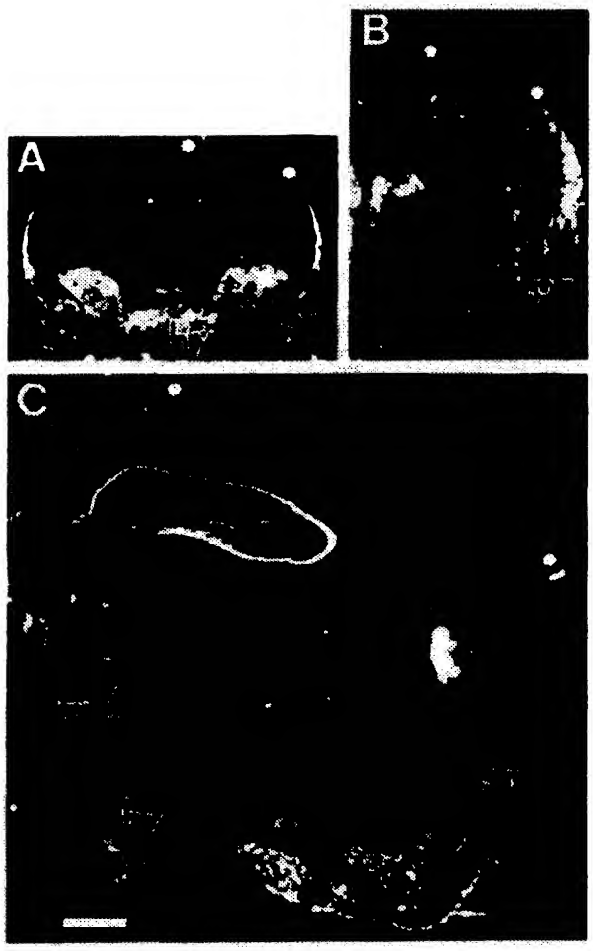

Figure 3. Darkfield Photomicrographs Illustrate the Specific Distribution of *lamp* Transcripts in the Developing and Adult Rat Brain

(A) Coronal section through the forebrain at E16 shows intense hybridization in the limbic perirhinal cortical region (pr) and hypothalamus (hy) and low signal in dorsal sensorimotor cortex (between asterisks).

(B) At the end of gestation (E20), the expression of the *lamp* transcript is high in the perirhinal region of cortex (pr) and sparse in the dorsal, nonlimbic cortex (between asterisks). The developing hippocampus (h) and midthalamic region, including the mediodorsal nucleus of thalamus (md), also exhibit intense hybridization.

(C) The specific expression of *lamp* transcripts remains in the adult brain, with intense hybridization signal in perirhinal cortex (pr), amygdala (a), hypothalamus (h), and medial thalamic region (md). Sparse signal is detected in sensorimotor cortex (between asterisks). Hybridization performed with riboprobes that spanned areas with high homology to *obcam* and *neurotrimin* resulted in patterns that overlapped with LAMP immunostaining, but also included areas lacking LAMP, such as olfactory bulb (data not shown).

Bar, 1 mm.

LAMP is a Selective Adhesion Molecule

To examine the functional properties of LAMP, we produced the recombinant LAMP in a mammalian system that is capable of faithful synthesis of the protein. Isolation of

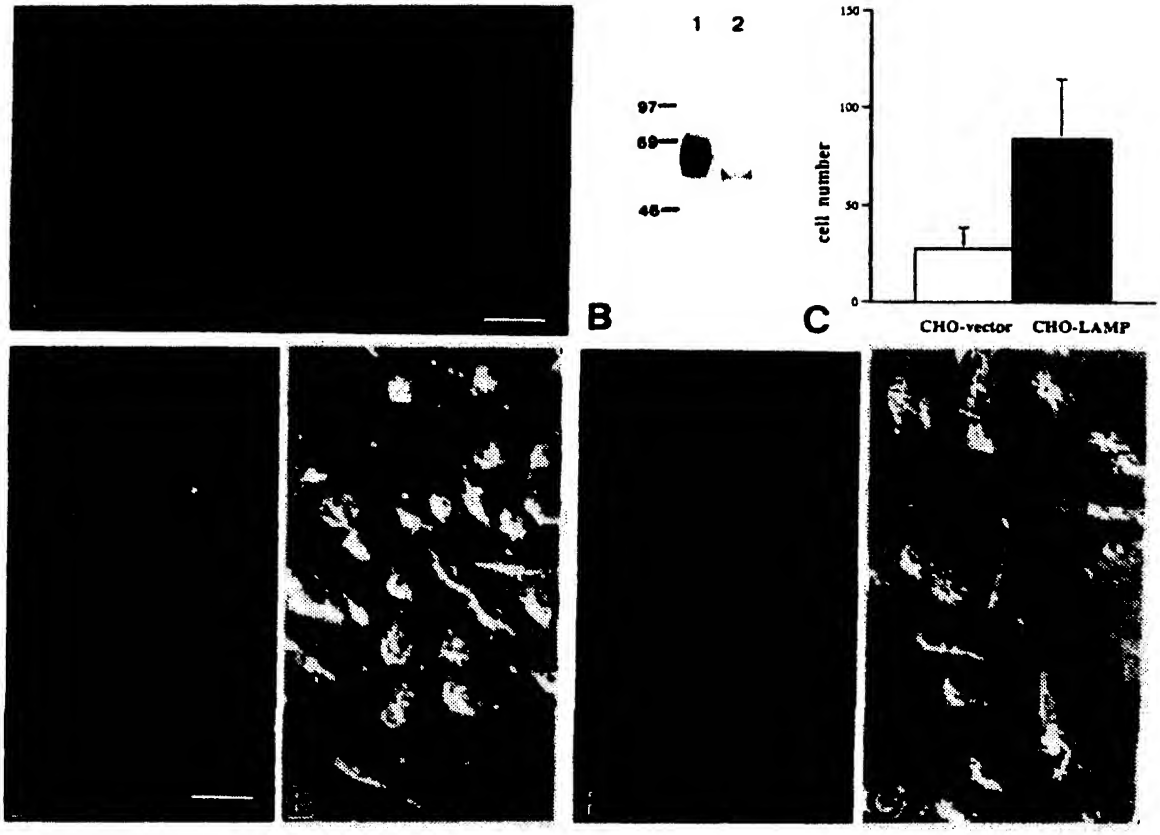

Figure 4. Recombinant LAMP Exhibits Selective Adhesive Properties

(A) Live CHO cells stably transfected with *lamp* construct (CHO_L) are immunoreactive with the antibody that recognizes the native form.

(B) Lane 1, native LAMP, released by PI-PLC treatment of hippocampal membranes, runs as a broad band between 64–68 kDa. Lane 2, the same treatment of the CHO_L cells releases all LAMP and runs as a more discrete band of approximately 55 kDa.

(C) Quantitative analysis of the specific binding of LAMP-coated Covaspheres to CHO_L-transfected cells compared with control, CHO vector-transfected cells (CHO_V). The number of cells in five equal fields per coverslip that had six or more beads bound were counted, and the mean of six coverslips in each category is displayed.

(D) Fluorescence micrograph depicts specific binding of LAMP-coated beads to the underlying CHO_L cells.

(E) Brightfield image of cells in (D).

(F) Fluorescence micrograph shows few LAMP-coated Covaspheres binding to the CHO_V cells.

(G) Brightfield image of cells in (F).

Bars, 5 μm (A); 20 μm (D–F).

stable transfectants yielded lines of CHO cells that express high levels of GPI-anchored LAMP, characterized by punctate immunoreactivity (Figure 4A) that is identical to the expression pattern of LAMP on the surface of cultured neurons. Treatment of the cells with PI-PLC results in release of the protein into the supernatant. The recombinant LAMP has an apparent molecular mass of 55 kDa (Figure 4B, lane 2), which is smaller than its native counterpart but larger than the polypeptide backbone. The recombinant protein in CHO cells thus appears to be partially posttranslationally modified consistent with reports that some glycosyltransferases are silent in host cells (Warren, 1993). Nonetheless, recombinant LAMP exhibits functional activity (see below). Native, immunoaffinity-purified LAMP exhibits homophilic binding (Zhukareva and Levitt, 1995). Indeed, we found that Covaspheres coated with native LAMP also can bind to its recombinant counterpart on the transfected CHO cells (Figures 4C–4G). This homophilic property suggests that LAMP may have the ability specifically to regulate growth of limbic neurons. To explore this hypothesis, embryonic neurons from LAMP-expressing hippocampus and perirhinal cortex and non-LAMP-expressing olfactory bulb and visual cortex were plated on a substratum of LAMP-transfected (CHO_L) or vector-transfected (CHO_V) CHO cells. Encountering recombinant LAMP, both limbic populations showed extensive neurite outgrowth within 24 hr, exhibiting well-differentiated morphologies and often extending long neurites (Figure 5). These same neuron populations always grew poorly on the CHO_V cells. Quantitative analysis of process lengths showed that the neurites on the CHO_L substratum are longer and with a substantial subpopula-

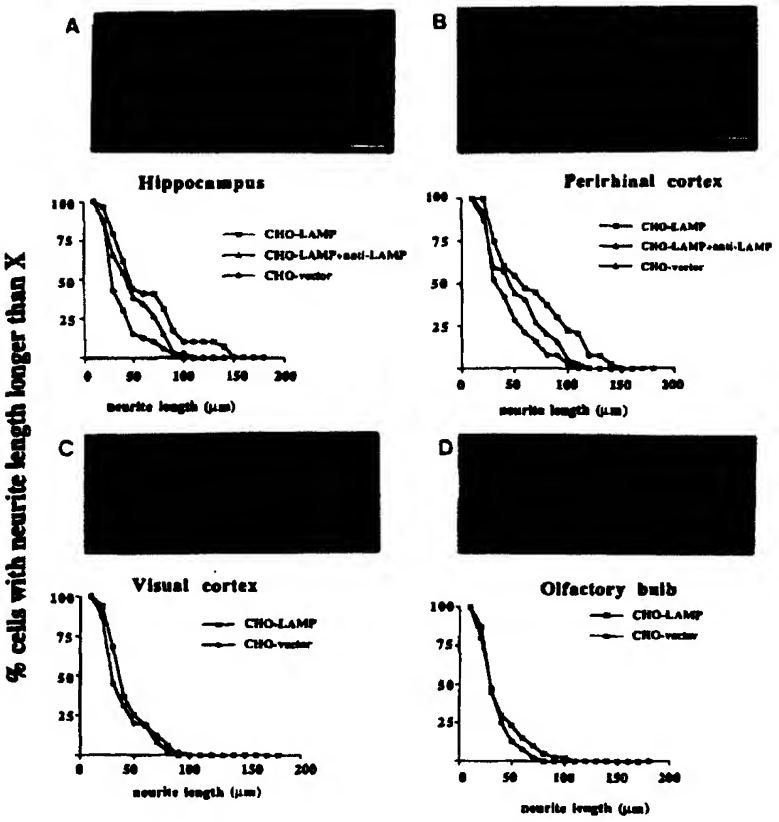

Figure 5. Analysis of Neurite Outgrowth of Limbic and Nonlimbic Primary Neurons on *lamp*-Transfected (CHO_L) and Vector-Transfected (CHO_V) Cells

(A) Hippocampal neurons grown on CHO_L substrate have longer neurites compared with growth on the CHO_V substrate. Anti-LAMP treatment of the cells plated on CHO_L eliminates the population of longest neurites (>100 μm) and reduces the number between 50 and 100 μm. Fluorescence photomicrograph above depicts well-differentiated MAP2-immunostained hippocampal neurons with long neurites on CHO_L substrate.

(B) Perirhinal limbic cortical neurons exhibit same specific outgrowth on CHO_L substrate as hippocampal neurons. In the photomicrograph, perirhinal neurons prelabeled with dye show similar neurite outgrowth as seen with MAP2 staining.

(C) Nonlimbic cells from occipital (presumptive visual) cortex exhibit the same pattern of neurite outgrowth on the two CHO substrates, with a noted absence of a neurite population greater than 100 μm on either substrate. Photomicrograph of MAP2-stained neurons on the CHO_L substrate reveals very short neurites. Magnification is same as in (A).

(D) Nonlimbic olfactory cells have similar neurite outgrowth profiles on CHO_L and CHO_V substrates. Note the absence of a neurite population greater than 100 μm. Photomicrograph depicts a group of dye-labeled cells with a few, very short neurites. Magnification is same as in (B).

Bars, 20 μm (A); 15 μm (B).

tion exceeding 100 μm (Figure 5). We further documented the specificity of the LAMP interaction by preincubating limbic neurons with functionally blocking LAMP antibodies prior to plating. This treatment resulted in a significant reduction of neurite length on the CHO_L cells (Figure 5). The effect was particularly evident for the population of longer neurites, which did not form following antibody exposure. The selective nature of LAMP-mediated outgrowth was illustrated by analysis of the behavior of nonlimbic cell populations. Both olfactory and visual neurons bound to the CHO_L substratum, but in contrast to their limbic counterparts, differentiated poorly and extended shorter neurites, which usually did not exceed 80 μm (Figure 5). In addition, neurites of the nonlimbic neurons grew almost identically on CHO_L and the control CHO_V cells, reflecting the absence of a specific, growth-enhancing interaction between the LAMP-expressing cell line and nonlimbic neurons.

LAMP Controls Axon Patterning in an Intrahippocampal Circuit

To study the effects of blocking LAMP function on a developing limbic circuit, we focused on a late-developing pathway in the hippocampus. Previous antibody perturbation in vitro showed that anti-LAMP can prevent axon targeting in the septo-hippocampal pathway (Keller et al., 1989). This circuit, however, develops prenatally, making it difficult to perturb in vivo with antibodies. In contrast, the excitatory glutaminergic mossy fiber projection of granule cells to pyramidal neurons begins to develop at birth and continues postnatally over a 3 week period in rats (Bliss et al., 1974; Amaral and Dent, 1981). Three features make this pathway amenable to manipulation with antibodies. First, we have shown that growing mossy fiber axons, growth cones, and their target pyramidal neuron dendrites express LAMP during development (Keller et al., 1989; Zacco et al., 1990). LAMP is maintained only on postsynaptic perikarya after synapse formation. Second, the ingrowth by mossy fibers occurs subsequent to the early, orderly innervation of pyramidal cell dendrites by commissural and entorhinal afferents. Third, the developing mossy fibers, which can be visualized most readily and specifically with the Timm histochemical stain, navigate in a stereotypic manner along a well-defined pathway that leads to the innervation of a discrete zone in the regio inferior of the hippocampus, the proximal portions of the apical dendrites of the pyramidal neurons (Bliss et al., 1974; Gaarskjaer, 1985; Ribak and Navetta, 1994). We injected animals intraventricularly four times during the first postnatal week either with control IgG, an anti-L1 monoclonal antibody (Sweadner, 1983) that binds to the membrane of developing axons, or anti-LAMP. Most animals were analyzed at the approximate midpoint of development of the mossy fiber pathway, postnatal day (P) 9–12. When exposed to either nonspecific IgG or anti-L1, the mossy fibers projected normally, with little or no growth through the stratum pyramidale or stratum oriens (Figures

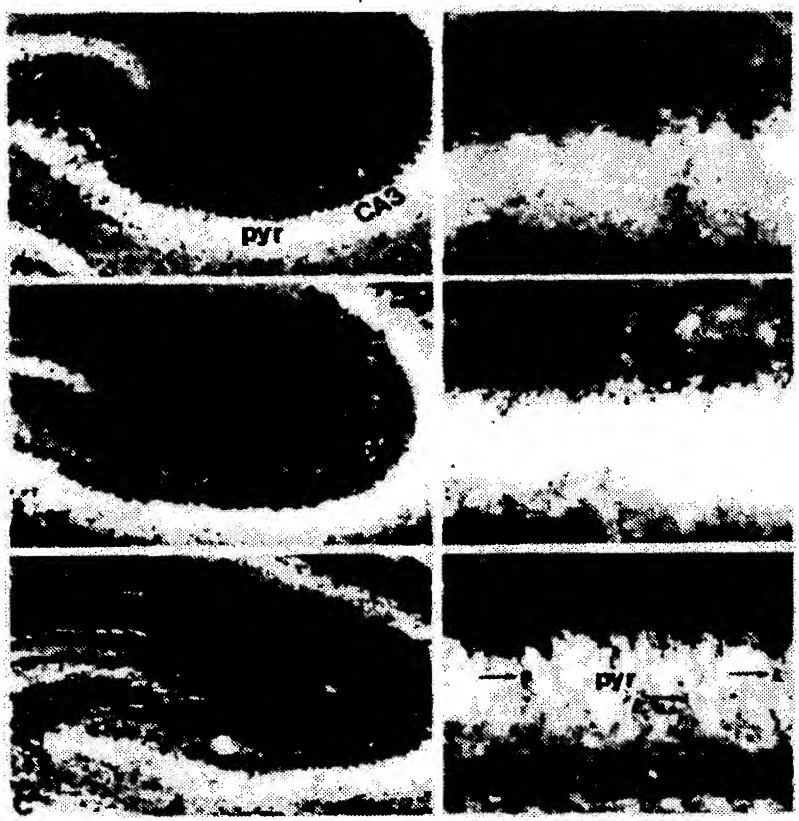

Figure 6. Direct Treatment of Postnatal Rats with Anti-LAMP Administered Intraventricularly on Alternating Days Results in Aberrant Growth of the Mossy Fiber Projection in the Hippocampus

Fibers visualized with the Timm stain in horizontal sections through the hippocampus. Low power photomicrographs of hippocampus from P9 rats injected with control IgG (A), anti-L1 (B), and anti-LAMP (C).

(A and B) The control and L1 antibody treatments result in a normal pattern of darkly stained suprapyramidal mossy fibers (smf), located in a tightly fasciculated zone within the stratum lucidum of CA3. Note the absence of fibers and boutons in the pyramidal cell zone, beneath in the stratum oriens (or), or above in the stratum radiatum (rad).

(C) At this low magnification, the Timm-stained pattern appears more diffuse than normal, with histochemical product in both supra-and infrapyramidal regions.

(D and E) Higher magnification photomicrographs of CA3 pyramidal zone from (D) IgG control and (E) anti-L1 animals. Both exhibit a normal mossy fiber projection, which is tightly arranged along the proximal suprapyramidal zone.

(F) Photomicrograph through the hippocampus from a P9 animal injected with anti-LAMP. There is aberrant growth of Timm-stained fibers throughout the CA3 region. Note the boutons and extensive fiber network (arrows) in regions that normally never contain mossy fibers, including stratum pyramidale (pyr), stratum radiatum (rad), and the alveus (alv).

Bar, 100 μm (A–C); 30 μm (D–F).

6A, 6B, 6D, and 6E; Figure 7A). In rats treated with anti-LAMP, however, we observed marked changes in the developing mossy fiber pathway. At low magnification, the developing projection appears uncharacteristically diffuse (Figures 6C and 6F; Figure 7B). Detailed examination at higher magnification, at which individual processes and varicosities can be seen, shows that misdirected axons traverse through the stratum pyramidale and oriens, as well as across the entire plexus zone of the stratum radiatum. In many animals, misrouted axons even entered the alveus, a pathway that normally carries fornix and commissural axons. Quantitative analysis comparing the normal and perturbed growth was performed in stratum pyramidale. In control animals, the calculated area fraction value is 0.05 ± 0.002, indicating that 5% of the area analyzed is occupied by Timm-stained profiles. Anti-LAMP treatment results in an area fraction value of 0.29 ± 0.006, a 6-fold increase in the area occupied by the aberrant mossy fiber projections ($p < .001$; t test). Limbic pathways that form prenatally, such as the septo-hippocampal cholinergic projection, are normal in the animals treated with anti-LAMP. The LAMP perturbation in vivo thus produces a significant departure from the normal patterning of the hippocampal mossy fiber projection.

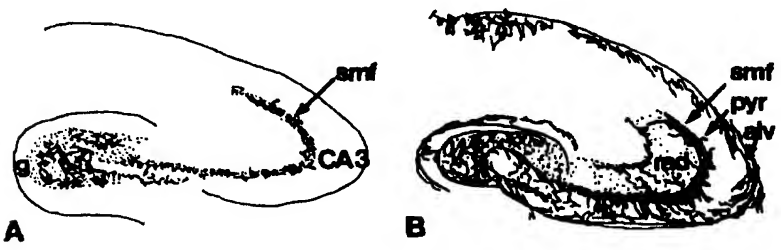

Figure 7. Camera Lucida Drawing of the Distribution of Mossy Fibers in the Normal or Anti-LAMP-Exposed Hippocampus at P9 from Two Animals

(A) Normal hippocampus; (B) anti-LAMP-exposed hippocampus.

The abnormal growth depicted here represents the more extreme phenotype following 1 week of anti-LAMP exposure. The restricted route of mossy fiber growth from the dentate gyrus into Ammon's Horn in the normal hippocampus is disrupted after anti-LAMP treatment, with fibers entering stratum radiatum (rad), pyramidal (pyr), and infrapyradimal zones, extending into the alveus (alv).

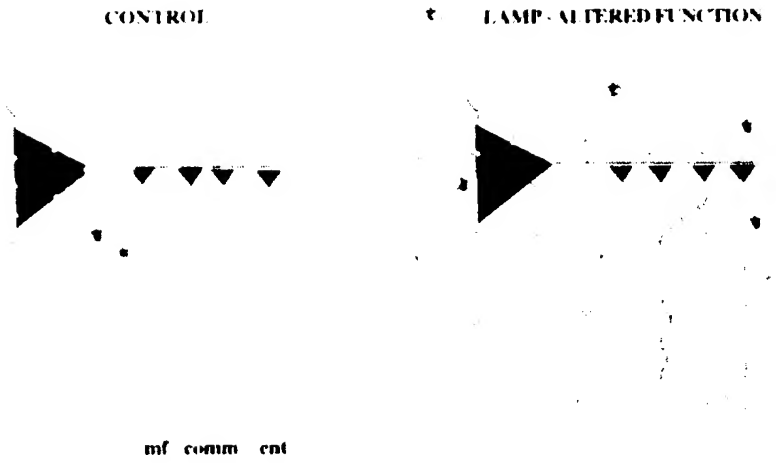

Figure 8. Summary Drawing Depicts Hypothesized Role for LAMP in Mossy Fiber Development

Mossy fibers (mf) normally fasciculate via homophilic LAMP interactions (yellow dots) in a restricted band of growth in the suprapyramidal zone. The earlier arriving commissural (comm) and entorhinal (ent) axons do not express LAMP upon synaptogenesis. In the LAMP-altered phenotype, the antibody (pink) causes mossy fiber defasciculation, resulting in atypical growth patterns into other zones and pathways. Note the absence of modification of the afferents that had arrived prior to the extended period of antibody exposure.

Discussion

Axon growth that occurs along defined pathways, and their subsequent invasion into specific targets to form functional circuits, has been proposed to be directed by unique molecular signals (Goodman and Shatz, 1993). In the invertebrate nervous system, distinct temporal and spatial expression of membrane-associated proteins is an important characteristic underlying the specificity of connections that form during development. Perturbation of the genes encoding these proteins has led to mutations in defined neural circuits (Goodman, 1994; Lin and Goodman, 1994). We have described the cloning of a new member of the IgSF, LAMP, and we have shown, using different experimental paradigms, that LAMP serves as an important mediator of axon patterning in the developing limbic system in the vertebrate brain.

Most CAMs exhibit a high degree of homology among forms of the proteins, indicative of conservation of function. LAMP is 99% homologous between rat and human, with transcripts of identical size encoding the protein, suggesting that all structural elements required for unique molecular recognition are retained. The homology between LAMP and most other CAMs of the IgSF is relatively moderate, probably reflecting structural features of the conserved sequences surrounding the cysteine pairs of the Ig domains required to form the β-pleated structure. In contrast, we found a strong relationship between LAMP and two other IgSF members that also have a three-domain loop structure, OBCAM and neurotrimin. All three are GPI anchored and exhibit very high homology within the first two domains. It is in the third loop that diversity among these subfamily members is expressed, and thus it is likely to represent the region that defines the functional specificity of each. Restriction analysis of the *lamp* gene reveals differences when compared with *neurotrimin*. This information is currently not available for *obcam*. In addition, the size of the transcripts encoding *lamp* are distinct from those identified with *neurotrimin* and *obcam* probes. Given the similarities between domains 1 and 2, it is likely that the three members of this subfamily arose through duplications that also resulted in specific structural divergence, providing diversity in expression patterns and function.

In a controlled environment, such as on CHO_L-transfected cells or a pure LAMP substrate (Zhukareva and Levitt, 1995), we showed that LAMP enhances outgrowth, but only for limbic neurons. The ability of antibodies to block this cellular response suggests that LAMP promotes homophilic binding. CAMs, however, can act through both homophilic and heterophilic mechanisms (Felsenfeld et al., 1994). Though heterophilic interactions between LAMP and another surface molecule also could contribute to the specificity that we report here using the in vitro assay, the relatively modest outgrowth of nonlimbic neurons on a LAMP substrate would require that a second molecule be expressed uniquely by limbic neurons. Under more complex conditions, such as in vivo or in explants, antibody perturbation does not disrupt cell differentiation or general fiber outgrowth, indicating that in the presence of other tropic molecules and CAMs, LAMP is not required for initiation or maintenance of axon growth. Rather, through homophilic interactions, LAMP probably participates in neuronal recognition that contributes to patterning of connections.

Though only correlative, the distinct anatomical patterns of LAMP expression during fetal development, shown immuncytochemically (Horton and Levitt, 1988; Ferri and Levitt, 1993) and here by in situ hybridization, are consistent with LAMP playing a role in the molecular recognition between groups of functionally related neurons. We initially tested this model using two different experimental paradigms, fetal tissue transplants and in vitro antibody perturbation. Using transplants of fetal cells, we were able to induce LAMP expression by grafted neurons that normally do not synthesize the protein (Barbe and Levitt, 1991). The resulting thalamo-cortical and cortico-cortical connections formed by the transplanted neurons with the host brain specifically reflect their new limbic phenotype (Barbe and Levitt, 1992, 1995). In explants, we showed that antibodies against LAMP prevented the invasion of the hippocampus by normal afferents, the septal cholinergic axons, resulting in a misrouted projection that grew

past their target (Keller et al., 1989). In the present study, we created a misrouted mossy fiber projection in the hippocampus in vivo using anti-LAMP, but failed to modify the developing projection with an anti-L1 monoclonal antibody. Similar to the results using explants, we did not find that anti-LAMP caused a reduction in fiber outgrowth or a major defect in the general direction of outgrowth. Rather, the mossy fibers grew in a less restricted fashion, extending through hippocampal zones that they normally never enter (Figure 8). Clearly, the mossy fibers use cues other than LAMP to extend from the dentate gyrus, but in the presence of LAMP antibodies, the fibers do not maintain the precision normally seen in this well-organized route. Imprecision in the development of the mossy fiber projection is reminiscent of mutations produced recently in Drosophila (Lin and Goodman, 1994). Loss-of-function *fasII* mutations result in decreased fasciculation of axons, but *fasII* does not play a major role in controlling the general direction and extent of neurite outgrowth. In some animals exposed to anti-LAMP during the first week and then examined as adults, we found similarly that the altered mossy fiber projection recovered. The loss-of-function *fasII* and LAMP phenotypes also are comparable to that seen with neurotransmitter-induced down-regulation of axonal apCAM in Aplysia, which led to a decrease in axon fasciculation and an increase in axon branching (Peter et al., 1994). Given the distribution of LAMP on all developing pyramidal neurons and dendrites in the hippocampus, the selective guidance role of the protein in normal mossy fiber development may involve the facilitation of axon–axon interactions among LAMP-positive processes when granule cell neurites first form postnatally. The restricted growth of mossy fibers into the proximal dendritic field probably reflects a combination of the selective adhesion among late-developing, LAMP-expressing mossy fibers and the occupation of the more distal dendritic fields by earlier forming entorhinal and commissural inputs (Figure 8).

Models of molecular recognition have been postulated by Sperry (1963) and others (Goodman et al., 1984; Jessell, 1988). Together with CAMs that are found ubiquitously in the vertebrate brain, developmental specificity is likely to be achieved through the early, selective expression of different classes of molecules. In this classic model, restricted synthesis of repulsive and attractive tropic molecules, such as collapsin, netrins, and CAMs (Kennedy et al., 1994; Serafini et al., 1994; Tessier-Lavigne, 1994), participate in pathway choices that subsequently lead to the involvement of selectively expressed adhesive proteins, such as LAMP, in target recognition. Defects in the tropic class would likely result in misguided axons that never reach their target, as seen in the netrin homolog *unc-6* mutation in C. elegans (Hedgecock et al., 1990; Ishii et al., 1992). We suggest that the absence of a putative targeting molecule like LAMP would produce somewhat more subtle defects, in which basic pathways form normally but contain growing fibers that bypass or grow through or around their normal fields of termination. In this context, the disrupted LAMP phenotype reported here exhibits features similar to the reported abberant mossy fiber outgrowth following induced epileptic activity in the adult hippocampus (Houser et al., 1990). It is possible, therefore, that regulation of LAMP expression by physiological activity, as shown for apCAM, will affect adult limbic circuits. The cloning of LAMP provides us with an opportunity, through molecular genetic strategies, to define further the contribution of this and related IgLONs to the guidance of developing axons and remodeling of mature circuits in the limbic system.

Experimental Procedures

Cloning and Sequencing

LAMP was affinity purified from adult hippocampal membranes (Zacco et al., 1990), run on SDS-PAGE, and electroblotted onto PVDF membranes in preparation for peptide microsequencing (Matsudaira, 1987). Bands were sequenced on an Applied Biosystem 470A gas phase sequencer equipped with a 120A on-line PTH analyzer (Henzel et al., 1987). An adult rat hippocampus cDNA library constructed in the λgt11 expression vector (Clontech) was screened with ^{32}P end-labeled, degenerate oligonucleotide probes derived from two distinct regions of the N-terminal amino acid sequence of LAMP, VRSVDFNRGTDNITVRQGDTA. Group 1 was GAYTTYAAYCGIGGIACIGAY and GAYTTYAAYAGRGGIACIGAY; group 2, ATHACIGTICGICARGGIGAY and ATHACIGTIAGRCARGGIGAY; R = A/G, Y = C/T, H-A/C/T I = inosine. Plaque purified clones that rendered positive for both probes had their cDNA inserts amplified by PCR (30 cycles: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min) using forward and reverse lambda primers (Promega) and were subcloned into pCR II vector (Invitrogen). Human cerebral cortex cDNA (1 ng; Clontech) was amplified by PCR using a combination of primers complementary to the rat *lamp* nucleotide sequence. PCR products were subcloned into pCR II vector (Invitrogen) or pGEM-T vector (Promega). The nucleotide sequence was determined, on both strands, by the dideoxy chain termination method (Sanger et al., 1977). Unless otherwise indicated, all standard procedures were performed essentially as described by Sambrook et al. (1989) and Hockfield et al. (1994).

Northern Blot Analysis

Total cellular RNA was isolated from adult, Sprague-Dawley rats, and the poly(A)$^+$ RNA fraction was purified using the PolyATract mRNA isolation system (Promega). Poly(A)$^+$ RNA (3 μg) was separated on 1.5% (see Figure 2A) or 1.2% (see Figure 2B) agarose–formaldehyde gel, transferred to a nylon membrane (Nytran, Schleicher and Schuel), UV cross-linked, and hybridized overnight under stringent conditions with ^{32}P-labeled RNA probes. Antisense probes were transcribed in vitro using T7 RNA polymerase from *lamp* cDNA template linearized with BalI (nucleotide 464–1238), while control sense probes were prepared with SP6 RNA polymerase from a template digested with NarI (nucleotide −55–471). The oligonucleotide probe (nucleotide 918–970) was labeled by the addition of a ^{32}P poly(A) tail.

In Situ Hybridization

Tissue sections fixed with 4% paraformaldehyde were hybridized with the 53-mer oligonucleotide probe labeled by the addition of ^{35}S poly(A) tail. Hybridization was performed at 58°C, with probe concentration at 10^6 cpm/ml of hybridization buffer (50% formamide, 10% dextran sulfate, 0.2 M NaCl, 1 × Denhardt's solution, 10 mM Tris, 1 mM EDTA). High stringency posthybridization washes included 1 hr in 1 × SSC at 60°C. No signal was detected on sections hybridized with the sense oligonucleotide probe.

Cell Transfections and Bead Binding Assay

The rat *lamp* cDNA was subcloned from pCR II into the EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen). CHO cells were transfected with 10–15 μg of pcDNA3–*lamp* using calcium phosphate precipitation. Transfectant clones were selected with 400 μg/ml active G418, and positive colonies were subcloned by limiting dilution. CHO cells transfected with pcDNA3 were used as negative controls. For localization of recombinant LAMP, live cells were incubated with mouse anti-LAMP, washed four times with DMEM/10% FCS, incubated with FITC-conjugated donkey anti-mouse following fixation with

4% formaldehyde, and mounted in glycerol/PBS with 5% propyl gallate. Fluorescent Covasphere beads (Duke Scientific) were incubated for 1 hr with PI-PLC-released, affinity-purified LAMP from hippocampal membranes. The coated beads were sonicated for 5 min immediately prior to plating on monolayers of transfected cells. Incubation continued for 30 min, followed by brief washes in culture medium, and then fixation in formalin solution. Counts of cells with more than six beads bound were made on each coverslip by examining five fields spaced 2 mm apart.

Neuronal Cultures on Tranfected Cells

Primary neurons from E16 embryos were prepared as described previously (Ferri and Levitt, 1993; Zhukareva and Levitt, 1995). Cells were resuspended in DMEM, and in some experiments, were labeled with lipophilic dye PKH26 (Sigma). Cells were plated in DMEM/10% FCS at the density 5 × 10^5 cells/ml on monolayers of CHO-transfected cells. After 48 hr in culture, coverslips were fixed with 4% formaldehyde, mounted, and examined under a fluorescence microscope. In some experiments in which cell prelabeling was not done, cultures were fixed and stained with the neuronal marker anti-MAP2, as described previously (Ferri and Levitt, 1993; Zhukareva and Levitt, 1995). Quantitative analysis was performed on dye-labeled and MAP2-stained cultures. The length of the longest neurite of 10–15 process-bearing cells, selected randomly, was measured on each of six coverslips prepared for each category in three different experiments. A Bioquant Image Analysis system was used to digitize and measure the images. The observer was blind as to the neuron population and substrate. Each point on the graph represents the percentage of total cells with neurites longer than X.

Antibody Application and Timm Stain

Sprague-Dawley rats (n = 34) were used. Anti-LAMP (n = 15) and control IgG (n = 14) anti-paramyosin (both IgG2a isotype) were affinity purified from hybridoma supernatant on a Protein A column. Anti-L1 (n = 5) was purified using a Protein A column and the MAPSII buffer system (Biorad) from ascites fluid (a gift of Dr. Jane Dodd, Columbia University College of Physicians and Surgeons), produced using the ASCS4 hybridoma cell line (Sweadner, 1983). Fab' fragments of all antibodies were made by digestion on immobilized papain (Pierce). Pure fragments (10 μg) were injected into the cisterna magna using a 32-gauge needle on P0, P2, P4, and P6. Animals were sacrificed on P9 by transcardial perfusion with 4.9% sodium sulphide (Na_2S) in phosphate buffer (pH 7.4). Brains were postfixed in Carnoy's solution with 1.2% Na_2S for 24 hr. Paraffin sections from control, anti-L1, and anti-LAMP treated groups were prepared for mossy fiber staining using the Timm method (Haug, 1973). For quantitative analysis, sections from control, L1, and LAMP antibody treated brains (n = 4 for each group) were stained in parallel and different subfields analyzed for density of innervation using the Bioquant OS/2 image analysis system. Six sections per brain at regular intervals were measured, with three fields per section measured.

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